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Ascorbate—cholesterol—lecithin interactions: factors of potential importance in the pathogenesis of atherosclerosis[1]

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In recent years, there have been many efforts to reduce the incidence of atherosclerosis, the leading cause of death in this country. Most of these efforts have involved programs designed to lower the blood cholesterol by one means or another, and most have met with only limited success. We have therefore undertaken a review of the subject of atherosclerosis for the purpose of determining potential new avenues of investigation that may prove fruitful in conjunction with programs already underway or in the planning stages. Our approach has been based on the following general concepts:

a) atherosclerosis, at least prior to the development of scarring, fibrosis and calcification, must be a reversible disorder;

b) it should not be considered an inescapable consequence of aging; and

c) it should be understood as a syndrome the pathogenesis of which is associated with both elevation of circulating lipids, and a variety of factors resulting in vascular injury.

As we must assume that injury and repair of the arterial wall are processes that go on continuously during life, disease will ensue only when the rate of the former predominates. If more injury is inflicted than can be repaired, or if the normal processes of repair are slowed down, disease will follow. Necessarily, then, schemes of prevention or therapy must aim at increasing the resistance to injury, at removal of injurious agents or factors interfering with arterial repair, or both.

A rather large volume of literature supports the hypothesis that vitamin C decreases susceptibility to vascular injury. There is also evidence that both vitamin C and certain unsaturated lecithins participate in the mobilization and excretion of cholesterol. The purpose of this article is to review the role of ascorbate in cholesterol metabolism and in the maintenance of the intercellular ground substance together with the role of the lecithins in cholesterol mobilization. It is our belief that the available scientific evidence clearly justifies, and indeed calls for, a carefully controlled evaluation of the effects of vitamin C and the lecithins on atherosclerosis.

General observations

A review of the literature has revealed a number of interesting observations in the past relating vascular disorders with ascorbic acid. For example, from October 1944 to February 1946, an experiment designed to determine the vitamin C requirements of humans was conducted at the Sorby Research Institute at Sheffield, England. The results of this study, reported by Sir Hans Krebs (1), are unique because 2 of the 10 young human volunteers (aged 21 to 34 years) who were given a scorbutogenic diet became suddenly severely ill with obvious cardiac emergencies requiring immediate interruption of the experiment and hospitalization. One developed intense substernal pain, dyspnea, cyanosis, shock, and elevation of S-T segments in the electrocardiogram. A few days later, another volunteer complained

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of sudden constrictive pain in the chest. A systolic murmur and electrocardiographic evidence of a partial heart block were detected. These obviously serious events occurred in the absence of signs of overt scurvy. The physicians of the eighteenth century were aware of similar life-threatening complications of mild scurvy. Lind, in 1757, wrote . . . "persons that appear to be but slightly scorbutic are apt to be suddenly and unexpectedly seized with some of its worst symptoms. Their dropping down dead upon an exertion of their strength, or change of air is not easily foretold" (1). That the underlying mechanisms of vascular injury in scurvy and in atherosclerosis might have many things in common is an hypothesis postulated and explored in the 1950's by Willis and his collaborators (2–5). He began by inquiring about the nature of the earliest lesions of atherosclerosis and concluded, as Virchow did in 1856, that it is a disturbance of the intercellular ground substance of the arterial intima, localized at points of mechanical stress (2). Osborn (6) in his study of the *Incubation Period of Coronary Thrombosis* concurred, and stated " . . . the first deviation from the ideal normal structure is a mucoid infiltration of the intima . . . the lipid lesions are appreciable later in development than the mucoid lesions." Willis recognized the similarity of these early lesions of atherosclerosis with those of scurvy which are, basically, alterations of mucopolysaccharide biosynthesis resulting in a relative diminution of the sulfated polymers. The latter are largely replaced by nonsulfated mucopolysaccharides that are more "mucoid" in character (7–9). Furthermore, it was realized that, as is the case in atherosclerosis, the localization of the vascular lesions in scurvy was also dictated by mechanical stress. In spite of these similarities, to postulate a cause–effect association between scurvy and atherosclerosis was, and still is, obviously impeded by the gross difference in prevalence of the two disorders and most importantly, by the established dogma that absence of overt scurvy signifies that all is well with regard to ascorbate nutriture and metabolism. This latter view, although still predominant, is being challenged more and more often. The challengers espouse the view that, in the words of Szent-Gyorgyi "Scurvy is not the first sign of the deficiency but a premortal syndrome, (and that) for full health, much higher amounts of ascorbate than those required to prevent scurvy are needed" (10).

A possible role for vitamin C in the prevention of atherosclerosis

From the foregoing discussion, it is apparent that vitamin C is concerned with the maintenance of normal vascular function, and that deficiency of it may cause vascular disease. For this concept to assume significance regarding the pathogenesis of atherosclerosis one must reconcile the fact that overt scurvy is of rare occurrence, whereas atherosclerosis is virtually endemic. The hypothesis that vitamin C deficiency contributes to the development of atherosclerosis implies that a prescorbutic state, rather than being rare, as scurvy is today, must be of quite common occurrence. It is this presumably frequent syndrome of subclinical vitamin C deficiency that would play a role in the pathogenesis of atherosclerosis. For this unorthodox view to win acceptance, it is necessary to show conclusively that:

1) Atherosclerosis, detectable in man or induced in experimental animals consuming adequate amounts of ascorbic acid to prevent scurvy, is worsened by ascorbate deprivation or restriction.

2) Supplementation of ascorbate intake beyond the level necessary to prevent scurvy should improve existing atherosclerotic lesions or prevent the development of new ones, or both.

3) No undesirable side effects ought to occur at the levels of ascorbate administration required to satisfy condition *2*.

There is considerable experimental evidence in support of the above postulates.

The use of an experimental model of chronic ascorbate deficiency obtained by restricting (but not eliminating) the ascorbate intake of guinea pigs allowed Willis (2) to demonstrate elevations of plasma cholesterol levels and appearance of histopathological lesions of atherosclerosis that were not observed in a control group fed the same diet but supplemented with larger amounts of ascorbic acid. The most effective atherogenic diet was an ascorbate-free, cholesterol-supplemented ration that produced atherosclerosis in all the guinea pigs that consumed it. Willis concluded that " . . . any factor disturbing ascorbic acid metabolism either *systemically* or *locally* results in

ground substance injury with subsequent lipid deposit." The intriguing idea of a localized ascorbate depletion limited to the arterial wall was explored by determining the ascorbate content of human aortas obtained at autopsy from three groups of patients: *1*) cases of sudden traumatic death, *2*) routine hospital autopsies, and *3*) patients who died after being on supplemental ascorbate for some time prior to death. The results obtained (5) showed the lowest levels of arterial ascorbate (7 out of 20 reported as zero) in the arteries of the routine hospital autopsies. The highest values were found, as expected, in the group supplemented with vitamin C prior to death, with the "sudden death" group falling somewhere in between. Not one of the aortas in these two groups was reported as having zero ascorbate. It is noteworthy that none of the patients constituting the "routine hospital autopsies" group had been diagnosed as having scurvy, and yet, the ascorbic acid concentration of their arteries was often undetectable. This localized arterial wall depletion of ascorbate occurred primarily in regions subjected to mechanical stress. Furthermore, the local ascorbate depletion seemed related to the duration and severity of the patient's illness.

Saroja et al. (11) have shown similar localized arterial wall deficits of ascorbic acid in guinea pigs fed estrogenic compounds. Importantly, the reduction of the ascorbate content of the arteries was much more pronounced than a concomitantly observed reduction of plasma ascorbate.

The role of systemic factors disturbing ascorbic acid metabolism and requirements is often ignored though extensively documented. Many drugs result in increased requirements of ascorbate. Among these are aspirin (12) barbiturates, paraldehyde, the hydantoins (13–15), aminopyrine, antipyrine (16), ether (17), and a large variety of carcinogenic compounds found as atmospheric pollutants (18–20) or as components of cigarette smoke (21). These increased requirements have been demonstrated by augmented levels of urinary elimination and lower plasma ascorbate levels, or by greatly increased rates of synthesis of ascorbate in experiments with rats which are animals that can synthesize their ascorbic acid (13–15, 20). Pelletier (21) has reported that cigarette smokers have lower levels of serum ascorbate and lower levels of ascorbate excretion in the urine after a test load than nonsmokers. His data indicate that cigarette smoking, one of the most important risk factors in atherogenesis, imposes increased requirements of vitamin C in man. The extent to which these factors contribute to chronic ascorbate deficiencies among the smoking, drug-oriented dweller of modern polluted cities cannot be precisely assessed, but must of necessity be significant. Additional consideration must be given to the trend in this country toward the consumption of highly processed "convenience" foods and other items having a low ascorbate content.

Ascorbate supplementation has been shown to reverse atherosclerotic lesions resulting from a prior period of vitamin C deprivation in guinea pigs (3). Even more interesting, serial, bilateral, femoral arteriographic studies of patients with clinical manifestations of generalized atherosclerosis have been used to follow the fate of individual atherosclerotic plaques and to assess the effect of ascorbate supplementation (4). The results obtained showed partial regression of plaques in 6 out of 10 ascorbate-treated patients (receiving 1.5 g/day), whereas none of 6 untreated patients improved.

Vitamin C deprivation, maintained for a period of time not long enough to produce scurvy, provides another experimental design to study the consequences of lack of ascorbate that precede the development of overt scurvy. Fujinami et al. (22) maintained guinea pigs for only 2 weeks on a scorbutogenic diet supplemented with coconut oil or ascorbic acid, or both, in various combinations. At the end of the 2-week experiment none of the animals was scorbutic; however, elevations of plasma cholesterol, cholesterol esters, phospholipids and nonesterified fatty acids were demonstrated in all those receiving no supplemental ascorbate. The ascorbate-deprived, coconut oil-supplemented group fared the worst. Addition of ascorbate returned the values to normal even in the presence of supplemental coconut oil. Of great significance was the finding that plasma levels of ascorbate in guinea pigs receiving both ascorbate and coconut oil remained low, strongly suggesting that the requirements for vitamin C may vary in relation to the content of fat in the diet in a manner similar to that in which thiamin requirements parallel the carbohydrate intake.

The experimental model of chronic ascorbate

deficiency in guinea pigs has been refined and standardized by Ginter and collaborators (23, 24). Desaturation of tissues by a scorbutogenic diet for a 2-week period is followed by a maintenance daily dose of ascorbic acid of 0.5 mg. Male guinea pigs maintained for approximately 4 months on this regimen weighed the same as a control group who received supplemental ascorbate (10 mg/24 hr).[2] The depleted animals had blood and liver cholesterol levels significantly higher than the control (171 versus 118 mg/100 ml in blood and 627 versus 456 mg/100 g in liver). An intriguing observation reported in this paper is that in female guinea pigs, 85 days of hypovitaminosis C did not increase the cholesterol levels of blood and tissues.

The mechanism responsible for the elevation of cholesterol in the ascorbate-deficient animals is said to involve an impaired conversion of cholesterol to bile acids (25). Acutely scorbutic guinea pigs injected with 26-^{14}C-cholesterol (the label is present in the side chain that is cleaved and metabolized to CO_2 on conversion of cholesterol to bile acids) eliminated subnormal amounts of $^{14}CO_2$ in their breath. Stimulation of cholesterol oxidation occurred 24 hr after injection of ascorbate, paralleling in time the appearance of cytochrome P-450 in liver microsomes. These results suggest that the effect of vitamin C in cholesterol oxidation is mediated by cytochrome P-450 which would be synthesized in response to administration of ascorbate. It is worth recalling here that the conversion of cholesterol to bile acids involves two hydroxylations (at carbon atoms 7 and 12) believed to precede the cleavage of the side chain. These hydroxylation reactions, as well as others in which ascorbic acid participates (such as the hydroxylation of proline, lysine, *p*-hydroxyphenyl pyruvate, barbiturates, and hydantoins, as well as a large number of other drugs and carcinogens) involve the cytochrome P-450-containing enzyme systems of liver microsomes.

In a recent paper, Ginter et al. (26) have shown conclusively that there is indeed a decreased rate of transformation of cholesterol to bile acids, not only in acutely scorbutic but also in chronically ascorbate-deficient guinea pigs.

The studies reviewed thus far have dealt primarily with experiments involving guinea pigs, which, like man, lack the ability to synthesize ascorbic acid. When animal species capable of ascorbate biosynthesis (such as rats and rabbits) are employed in the production of experimental atherosclerosis, the beneficial effects of ascorbate supplementation can also be demonstrated. This seems to be an important consideration because, in these animals, the production of scurvy is impossible. In our view, it furnishes strong evidence for the contention that some beneficial effects can be elicited with ascorbate supplementation exceeding the minimal scurvy-preventing levels, which in these species are always provided by their own biochemical machinery. Zaitsev et al. (27), Myasnikov (28), and later Sokoloff and associates (29), studying both rabbits and rats fed cholesterol-supplemented diets with and without ascorbate additions, demonstrated that the vitamin C-enriched diets largely prevented the elevation of blood cholesterol. The blood cholesterol values obtained by Sokoloff et al. with rabbits fed the experimental rations for 8 months were as follows: control group (no cholesterol added), 88.5 mg/100 ml; cholesterol-supplemented group, 1,234 mg/100 ml; and cholesterol + ascorbate group (150 mg/kg of ascorbate), 308 mg/100. Essentially identical results were found employing rats as the experimental animal. Of added interest is the finding that lipoprotein lipase activity of plasma which decreases as the cholesterol levels go up, was maintained at normal levels in the ascorbate-supplemented group. This finding has been confirmed by Fujinami et al. (22).

The effects of ascorbate supplementation on the circulating cholesterol levels of man are clouded by controversy. Myasnikov (28) and Sedov (30) reported a lowering of cholesterol levels when ascorbate in high doses was given to hypercholesteremic patients. Anderson et al. (31) found no effect on the cholesterol levels of normocholesteremic individuals. Samuel and Shalchi (32) studied 14 cases of hypercholesteremia treated with high doses of ascorbate and could show a significant lowering of cholesterol in only one. Sokoloff et al. (29) report mixed results, some of their patients responded with dramatic drops, whereas others showed no change. Ginter et al. (33), studying a

[2] One must note that scurvy in the guinea pig is regularly produced in only 3 to 4 weeks of ascorbate deprivation; at this time the animals suffer precipitous weight losses and soon die.

group of individuals from a region where a seasonal deficit of vitamin C is known to occur, reported significant lowering of blood cholesterol levels following administration of 300 mg ascorbate/day. Hodges and co-workers (34), in a study of experimental scurvy in man, observed a tendency for the serum cholesterol to rise in some subjects but did not consider the change statistically significant (34). In a subsequent study, Hodges et al. reported that during ascorbic acid depletion (35), the serum cholesterol declined in a group of subjects who were consuming a diet rich in polyunsaturated fats and virtually devoid of cholesterol. It is not possible to assess the effect of weight loss as a cholesterol-lowering factor in these subjects. Evidently, more work is required to settle this important matter.

A recent paper by Knox (36) presents the results of correlation analysis between mortality ratios of ischemic heart disease, cerebrovascular accidents (and other causes of death), and the intake of various nutrients. Strong negative correlations were found between ascorbate consumption and both ischemic heart disease ($r = -0.49$) and cerebrovascular disease ($r = -0.68$), the latter being the strongest negative association found in the study. This work is self-supporting in the sense that known risk factors of atherosclerotic disease, such as a high fat diet and high vitamin D intake showed positive correlations, whereas high dietary calcium gave a strong negative correlation coefficient in keeping with the known protective effect conferred by hard water (37).

The seemingly beneficial effects of high ascorbate consumption on the course of atherosclerosis may be due to one or more of the following biochemical mechanisms. Aside from the already discussed cholesterol-lowering effect mediated through an increased rate of conversion of cholesterol to bile acids, the formation of cholesterol sulfate (a water-soluble ester of cholesterol) from ascorbate sulfate, must be considered. Both of these substances have been identified in humans (38–40) and the sulfating ability of ascorbate sulfate has been amply substantiated chemically (41). Furthermore, it has been shown (42, 43) that administration of ascorbic acid and ascorbic acid sulfate to rats results in a twofold and a fiftyfold elevation, respectively, of the fecal excretion of cholesterol sulfate.

Other effects of ascorbic acid on the metabolism of sulfur-containing compounds can be of great significance in explaining the vitamin's purported role in the prevention of atherogenesis. As briefly mentioned before, a characteristic biochemical lesion of ascorbate deficiency seems to be a relative diminution of . . . the sulfated mucopolysaccharides (or glycosaminoglycans, abbreviated "GAG") of connective tissue. It has also been shown that nonsulfated GAG of the hyaluronic acid type obtained from atherosclerotic human aortas bind plasma lipoproteins and fibrinogen (44), although another GAG fraction of the same tissues having a high content of sulfate does not. These findings seem to indicate that an abnormal ratio of sulfated to nonsulfated GAG, perhaps resulting from marginal, relative, or even localized deficiencies of ascorbic acid (or derivatives thereof, e.g., ascorbic acid sulfate) could provide the altered ground substance on which the lipids accumulate as a secondary event in the arterial wall. It is worth mentioning that the content of nonsulfated GAG in the aortas of several animal species decreases together with their susceptibility to experimental atherosclerosis (45) and that the aorta of female cattle contains a lower amount of nonsulfated GAGs than their male counterparts, again in keeping with the higher susceptibility of males to arterial disease (45). Besides, numerous studies have been published showing beneficial effects on both human (46, 47) and experimental (48, 49) atherosclerosis resulting from the administration of sulfated glycosamino glycans.

In the light of this discussion, it is pertinent to consider the adequacy of the present recommended dietary allowances for ascorbate and the possibility of side effects associated with higher levels of intake. The *Recommended Dietary Allowances* (50) establishes 45 mg/day of ascorbate as the amount recommended for an adult man, or approximately 0.7 mg/kg of body wt. As pointed out by Stone (51), it is remarkable that, the recommended intakes of ascorbate on a per kilogram basis for other primates and for guinea pigs, as established by the Committee on Animal Nutrition in its publication *Nutrient Requirements of Laboratory Animals* approximates 10 mg/kg for monkeys and between 20 and 50 mg/kg for guinea pigs. The latter has been verified

recently by Yew (52). The gross discrepancies (a 10- to 50-fold differential) between the recommended allowances for man and other ascorbate-requiring animals are undoubtedly the result of having based the human requirements on the amount necessary to prevent scurvy in a healthy, resting young man (approximately 10 mg/day), whereas the animal figures are based on the amounts required for optimal health, including parameters such as growth rates, responses to surgical stress, wound healing rates, resistance to infection, and others (52). Bourne (53) and Pauling (54) have presented an intriguing evolutionary argument that places the amount of ascorbate that would be consumed by a vegetarian hominid at almost 2,300 mg/day, or close to 40 times the RDA, and nearly the same, on a per kilogram basis, as the amount recommended for monkeys and guinea pigs.

Since the publication in 1970 of the book *Vitamin C and the Common Cold* by Linus Pauling (54), there has been a resurgence of interest not only in the clinical effects of the vitamin but in its mechanism of action. There have now been at least three well-designed, independent, and carefully conducted studies all of which suggest the effectiveness of vitamin C in reducing the incidence and severity of upper respiratory infections (55–57). Although one may speculate as to whether the observed results are due to a nutritional or pharmacological mode of action, it now seems well-established that beneficial effects do occur. Coulehan et al. (57) are inclined to favor a local pharmacological action on the respiratory mucosa, but the evidence of Anderson et al. (55) suggests a more generalized benefit. In the context of the present review regarding atherosclerosis, the important point seems to be the demonstration of desirable effects at a level of intake exceeding the minimal amounts necessary to prevent scurvy. It seems reasonable to ask if similar levels of intake will be beneficial in other tissues, such as blood vessels.

Toxicity has not been observed with prolonged ingestion of ascorbate in amounts of 1.0 to 2.0 g/24 hr (54), although some concern has been expressed on theoretical grounds over possible ill effects at this level of intake. The possibility of oxalate stone formation in the kidney has been investigated experimentally on human volunteers and animals by Takenouchi and associates (58). Upon oral or intravenous administration approximating 3.0 g/24 hr of ascorbic acid, there was no significant increase of urinary oxalate. Fear of this potential complication seems therefore unwarranted. Prolonged use of ascorbic acid in large doses may be undesirable in certain situations such as hepatic cirrhosis, renal tubular acidosis, uremia, and hemolytic anemia due to glucose-6-phosphate dehydrogenase deficiency. However, we are not aware of any well-documented reports of ill effects ascribable to high doses of ascorbate, although there have been several large-scale studies. The paucity of adverse reports suggests that doses as high as 20 times the current RDA are well-tolerated by the vast majority of the population.

Possible relationships between lecithin and cholesterol in the etiology of atherosclerosis

Free cholesterol, which predominates in both early and advanced atherosclerotic lesions (59), is virtually a water-insoluble compound. Moreover, its ring structure cannot be degraded by the animal organism. It is true that ring opening occurs upon exposure of 7-dehydrocholesterol in the skin to ultraviolet light, but this involves only microgram quantities and is negligible in considering total cholesterol turnover. The consequence of these facts is that once cholesterol is deposited in a tissue, months may be required for its removal (69); furthermore, only a limited number of mechanisms seem to exist for mobilizing it. These include: *1*) transformation into a more water-soluble derivative, *2*) an alteration of the physicochemical properties of the solvent (plasma) to allow a greater uptake of the unmodified cholesterol, and *3*) phagocytosis.

The synthesis of cholesterol derivatives in the arterial wall takes the form of ester biosynthesis, the acid moiety of which is provided by a variety of fatty acids (with the exception of cholesterol sulfate mentioned before). The properties of the cholesteryl esters vary with the kind of fatty acyl moiety esterified to the sterol. Adams (61) has ranked the sclerogenic properties of free cholesterol and its esters in the following decreasing order: monounsaturated esters – free cholesterol – saturated cholesteryl esters – cholesteryl linoleate (18:2) – cholesteryl linolenate (18:3) – cholesteryl

arachidonate (20:4). Furthermore, the rate of resorption of cholesterol and cholesteryl ester implants is higher for the triene and tetraene cholesteryl esters. Clearly then, as polyunsaturated esters are the least sclerogenic and the easiest to remove from tissue deposits, their formation should be beneficial in both the prevention and resolution of atherosclerotic plaques. The origin of, and the mechanism of esterification to, cholesterol of the polyunsaturated fatty acids therefore assumes considerable significance. There is mounting evidence supporting the role of the enzyme lecithin:cholesterol acyltransferase (LCAT) as the main catalyst of cholesterol esterification (Fig. 1). This enzyme, which was discovered in plasma by Sperry (62) many years ago, catalyzes the transfer of a fatty acyl residue from the β-position of a lecithin to the hydroxyl group of cholesterol. Individuals who lack this enzyme develop hyperlipidemia and atherosclerosis at an early age (63). It is important to remember that lecithins, like triglycerides, constitute a large family of related compounds differing in the kinds of fatty acids esterified to positions 1 and 2 of the glycerol moiety. Obviously, not all lecithins will serve equally well as substrates for the LCAT enzyme, an important fact demonstrated in vitro by Murphy in 1962 (64). It follows therefore that only those lecithins bearing a polyunsaturated fatty acid in position 2 and serving as good substrates for the lecithin:cholesterol acyltransferase would promote the desirable synthesis of polyunsaturated cholesteryl esters and favor the removal of cholesterol deposits. Saturated lecithins would not do this. That such is indeed the

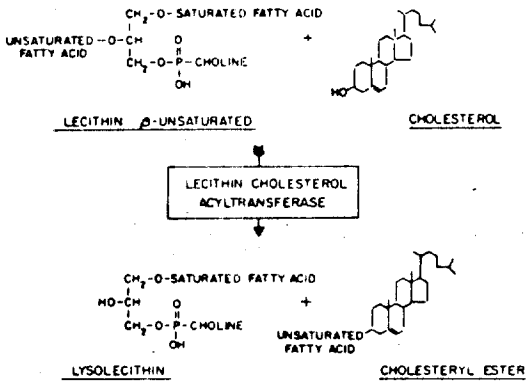

FIG. 1. Role of lecithin:cholesterol acyltransferase in cholesterol esterification.

case is indicated by experiments of Adams and co-workers (65) demonstrating that the administration of a polyunsaturated lecithin derived from soybean not only accelerated the resorption of cholesterol much more effectively than the relatively saturated lecithins from eggs, but in effect prevented the development of cholesterol-induced atheroma in the rabbit (66). The disappointing fact that in this species the lecithin had to be administered intravenously does not rule out the possibility that oral feedings of the soybean lecithin may be effective in humans. In this regard, it is most important that Bloom et al. (67) and Blomstrand (68) conclude that a highly significant amount of ingested lecithins is absorbed intact. The reports of beneficial effects of soybean lecithin supplementation in human atherosclerosis (69) lends direct support to the above contention.

It is indeed probable that the beneficial effects derived from a diet rich in polyunsaturated fatty acids may be obtained through their prior conversion in the body to 2-polyunsaturated lecithins with subsequent transfer of the polyene acid onto free cholesterol. Most important to note is that this mechanism would be entirely dependent on the ability of the body to synthesize the polyunsaturated lecithins from the polyunsaturated triglycerides provided in the diet. The requirements of the lecithin synthesizing enzymes may thwart this possibility. It is known (70, 71) that the enzyme which converts D-α,β-diglycerides to lecithins by reaction with cytidine-diphosphate-choline has a pattern of specificity for diglycerides such that some of them (i.e., α,β-dioctanoin, dilaurin, or dipalmitin) are not at all utilized in lecithin synthesis. The presence in the diet of a polyunsaturated triglyceride is therefore no guarantee that the desired 2-polyunsaturated lecithin would be formed from it. As mentioned earlier, it would make better sense to administer the preformed compound, particularly since lecithins are largely absorbed intact.

Rutenberg and Soloff (72) have presented experimental evidence to support the involvement of lecithin:cholesterol-acyltransferase in the mechanism responsible for removal of free cholesterol from the arterial wall. Their hypothesis is based on the work of Murphy (64) who demonstrated an equilibrium between free cholesterol of plasma and red blood cells; the

flux of free cholesterol from the cells to plasma increases as the free cholesterol of plasma becomes esterified. Cholesterol esters do not exchange with red cell cholesterol which is nearly totally free. Rutenberg and Soloff propose a similar equilibrium between plasma-free cholesterol and that of the arterial tissue. Increasing the esterification of the free cholesterol of plasma should allow more cholesterol to leave the arterial wall. Experimental support for this mechanism is provided by their work together with intriguing preliminary evidence of impaired cholesterol esterification in patients with myocardial infarction and chronic coronary artery disease (72). A deficiency of LCAT (or perhaps one form of LCAT) can be suggested as an etiological factor in atherosclerosis. In this regard the reported diminution of LCAT activity in plaques and complicated lesions as compared with fatty streaks (73) as well as the extensive atherosclerotic changes in the abdominal aorta of a patient with hereditary LCAT deficiency who died (74) neatly fits the scheme.

The physicochemical role of lecithins in the solubilization of cholesterol in aqueous media has great biological significance and has been the subject of some excellent recent publications. Even though the enormous complexity of biological fluids such as plasma cannot be faithfully reproduced by laboratory models, much has been gained by the study of these simplified systems. Small (75) in developing a classification of biological lipids based on their interaction in aqueous systems has shown that, even though cholesterol at 22 C is virtually insoluble in water, a one-phase lamellar liquid crystal is formed upon addition of lecithin. The maximum amount of cholesterol that can be incorporated into this phase being one molecule per molecule of lecithin. Of great importance again is the fact that the degree of unsaturation of the fatty acids of the lecithin greatly influences its ability to form a liquid crystal at the temperature of the body. Thus . . . "distearyl lecithin will not form a liquid crystalline phase at room temperature. However, at 70°C the hydrocarbon parts of the molecule melt and the crystalline phase transformation takes place. On the other hand di-linoleyl lecithin will form liquid crystals at 0°C in water" (74). Obviously, distearyl lecithin at body temperature will contribute little if anything to the solubilization of free cholesterol, whereas dilinoleyl lecithin would contribute significantly. Essentially the same type of results have been reported by Hofmann (76) and by Saunders and Wells (77).

It is worth emphasizing that none of the possible mechanisms adduced to support the role of unsaturated lecithins in the prevention of atherosclerosis are mutually exclusive. In fact, it is likely that more than one would enter simultaneously into play.

Summary

In this review we have attempted to focus attention on some of the factors involved in the injury–repair systems of the arterial wall as well as to factors favoring the deposition of lipid at sites of injury. No effort has been made to review factors such as mechanical stress and hypertension which have a rather well-established link to the development of atherosclerosis. Likewise, it has not been possible to consider in detail events such as radiation injury or exposure to toxic drugs and chemical agents which might produce localized damage to arterial walls. However, considerable evidence is cited emphasizing the importance of intercellular glycosamino-glycans in arterial wall metabolism and the important role of ascorbic acid in the formation of these materials. Evidence is available from studies involving both humans and experimental animals, that in ascorbate deprivation, there is an accumulation of the nonsulfated polymers in the arterial wall. Such mucoid material binds plasma lipids and fibrinogen with greater affinity than do the sulfated polymers. Ascorbic acid also appears to play a significant role in cholesterol metabolism. In a number of animal experiments and in some (but not all) human studies, ascorbate is reported to have a cholesterol-lowering effect. Thus, vitamin C seems to occupy a position of unique importance by virtue of its involvement in two systems: the maintenance of vascular integrity and the metabolism of cholesterol to bile acids.

This review describes certain aspects of cholesterol mobilization as related to β-unsaturated lecithins. These compounds, through the action of the enzyme lecithin:cholesterol acyltransferase, may play a key role in the formation of cholesterol esters and also aid in

cholesterol transport through the formation of water-soluble systems. Although vitamin C and the lecithins are being actively promoted and widely sold in health-food stores, there have been no carefully controlled scientific evaluations of either their effectiveness or safety in statistically significant groups of humans. It is our belief that the available evidence clearly justifies, and indeed calls for, such studies.

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QUANTITATIVE LIPID ANALYSIS BY COMBINED THIN LAYER AND GAS–LIQUID CHROMATOGRAPHIC SYSTEMS

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1. INTRODUCTION

The domain of lipids comprises substances ranging from simple fatty acids to the complex phospholipids and from steroids to certain fat soluble pigments and vitamins[1]. In the past, these compounds have been considered related mainly because of their

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mutual solubility in organic solvents and not necessarily because of other physical, chemical or biochemical characteristics. Many of them, however, occur together in lipoproteins and lipid membranes where they apparently form highly organized structures possibly of a liquid crystalline type[2]. Therefore, the study of the chemical composition and structure of natural lipid mixtures has become of great interest. It is hoped that the knowledge of complete lipid composition will help in the elucidation of the molecular relationships in lipoproteins and lipid membranes and will further the understanding of the phenomena governed by these physical systems.

Because of the chemical diversity of these substances, the major difficulty associated with any study of the chemistry or biochemistry of lipids has been that of isolation and separation into individual components[1]. The closely related solubility properties have made the purification of these substances by classical means virtually impossible. Within the past few years, however, new and improved procedures based upon the principle of chromatography have been forthcoming and have allowed a better fractionation and more complete identification. The chromatographic efforts have culminated in the development of combined or integrated thin-layer (TLC) and gas–liquid chromatographic (GLC) systems, which permit an essentially complete resolution and quantification of all components of natural lipid mixtures. The present review is restricted to the consideration of such integrated systems only, and does not constitute an attempt at a comprehensive review of either TLC or GLC techniques themselves, detailed accounts of which may be found elsewhere. The review includes a theoretical section in which the more obvious advantages and possible disadvantages of the integrated analytical systems are discussed.

2. THEORETICAL CONSIDERATIONS

In the integrated TLC–GLC system to be discussed, the preparative TLC step effectively replaces chromatography on silicic acid columns as a means of isolation of individual lipid classes. The experience gained from the work with the silicic acid columns, however, has been invaluable in the development of satisfactory TLC systems. Besides supplying information for an intelligent choice of developing and eluting solvents, adsorption chromatography on silicic acid columns has provided the reference separations necessary for appraising the success of the early TLC–GLC combinations. It is both informative and experimentally instructive to open the discussion with a brief consideration of the factors involved in developing the integrated TLC–GLC systems.

(a) *Advantages of the integrated systems*

In most cases, TLC results in better resolution in a shorter period of time than comparable column adsorption chromatographic techniques and has therefore become an indispensible tool in lipid analysis. The great advantages of TLC, however, are fully exploited only if it is combined with other chromatographic systems that provide complementary separations. For the analysis of lipids, GLC is superior to any other technique for detailed resolution and quantitative estimation of the small amounts of material conveniently obtained from analytical TLC plates.

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1. Complementary nature of separation

TLC is capable of fractionating lipids according to chemical classes and degree of unsaturation, as well as of resolving certain members of homologous series and some stereo and positional isomers[3]. In this respect it resembles chromatography on adsorption columns except that the separations on the thin-layer plates are more complete and more rapidly accomplished. Only a few mg of material are required for the separation and relatively crude extracts may be employed. TLC has proved to be especially useful for separations of classes of compounds which are either insufficiently volatile or too unstable for analysis by GLC.

Although complete separations of most lipid classes are readily accomplished by TLC, direct quantification from plates is difficult[4]. GLC is capable of the greatest sensitivity, highest resolution and accuracy in the analysis of fatty acid methyl esters, sterols and other low molecular weight lipids[5], but neutral glycerides[6] and steryl esters[7] can also be effectively determined. The glycerides and steryl esters are resolved on the basis of molecular weight only, and each GLC peak may represent a group of compounds of the same molecular size. The composition of these peaks may be determined following collection by preparative GLC[8] and analysis of the component acid and alcohol parts of the molecule by conventional GLC. The fatty acids after conversion into the methyl esters can be completely resolved on the basis of molecular weight, unsaturation and geometric or positional isomerism[5]. The GLC separations can conveniently be carried out at the nanogram level[9], although usually samples of a few micrograms are used. A single TLC spot can provide enough material for several runs in the gas chromatograph.

The subfractionations of lipid classes on the basis of unsaturation, provided by TLC on silver nitrate impregnated silica gel[10], are of great interest in the study of biochemical transformations of lipids. Although individual components are seldom obtained, each lipid class is broken down into simpler and more accurately defined mixtures than those obtained by conventional TLC. GLC is ideally suited for the quantification of the small amounts of material available from such analytical plates as well as for further resolution and/or collection of fractions of uniform molecular size from preparative silver nitrate separations. Some of the possibilities provided for the analysis of a complex lipid class, by the combination of TLC and GLC, are illustrated in Table 1 using natural lecithins as an example. The overall scheme appears to be suitable for the analysis of other phospholipids such as phosphatidyl serine, phosphatidyl ethanolamine and phosphatidyl inositol, all of which have been found to yield diglyceride acetates on acetolysis[11]. Analytical sequences utilizing combinations of TLC and GLC in the determination of triglyceride structure have yielded more accurate analyses than either of these methods used singly, or indeed than any other method of fractionation[12].

In addition to providing individual chemical classes of lipids for effective further separation and quantification by GLC, TLC also serves as a means of purification of the appropriate lipid components. It is thus seen that the combination of TLC and GLC is not only favorable from an analytical standpoint, but that it is a practical, feasible system which can facilitate and improve any lipid analysis regardless of whether or not the need for all the information potentially available is immediately required. As a result of the application of the integrated complementary analysis by GLC and TLC it becomes possible to determine the fatty acid composition and the

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TABLE I

SCHEME FOR COMPLETE ANALYSIS OF LECITHINS[13]

A	B	C
1. Isolation of pure lecithins by TLC.	1. Isolation of pure lecithins by TLC.	1. Isolation of pure lecithins by TLC.
2. Acetolysis or specific hydrolysis by phospholipase c (RENKONEN[11]).	2. Separation of lecithins by silver nitrate TLC (ARVIDSON[15]).	2. Separation of lecithins by silver nitrate TLC.
3. Separation of diglyceride acetates by silver nitrate TLC.	3. Isolation of saturates, monoenes, dienes and polyenes as diglyceride acetates.	3. Specific hydrolysis of original mixture and each subfraction by phospholipase a.
4. Analytical and preparative GLC of saturates, monoenes, dienes and polyenes (KUKSIS AND MARAI[13]).	4. Preparative GLC and specific lipase hydrolysis as in A.	4. Complete fatty acid analysis of the original mixture and each subfraction.
5. Specific lipase hydrolysis of diglyceride acetates of uniform molecular weight and uniform degree of unsaturation.	5. Complete fatty acid analysis of the original mixture and each subfraction as in A.	5. Reconstruction of the composition of the original mixture from fatty acid data* obtained in A, B and C.
6. Complete fatty acid analysis of the original mixture and each subfraction.	6. Reconstruction of the composition of the original mixture from fatty acid data*.	
7. Reconstruction of the composition of the original lecithin mixture from the fatty acid data*.		

* Previous knowledge of the reproducibility and reliability of the fatty acid data from each fraction is used and, if necessary, certain measurements are assumed to be absolutely precise so that other values can be calculated by difference. Simple normalization is not used unless there is no alternative.

chemical structure of individual members of each lipid class, except for positional isomers which must be determined by specific enzymic hydrolysis.

2. Ease of quantification

Since all the peaks obtained in the analytical gas chromatograph can be measured by means of internal standards, there is no need for quantification on TLC plates, provided complete transfers and recoveries have been made at each step. Although phospholipids as such cannot be recovered by GLC, they can be measured by this means as the diglyceride acetates[13] after acetolysis or as the fatty acid methyl esters following transmethylation[11]. The transformation of the phospholipids into the methyl esters of fatty acids[9] or diglyceride acetates[13] in the presence of the silica gel, avoids the necessity of obtaining complete elutions of the more strongly adsorbed original materials. It serves also as a means of determining the completeness of recovery when such is attempted. The addition of a carefully measured amount of an internal standard (usually a foreign fatty acid ester) to the lipid mixture to be analyzed by GLC, and subsequent quantification on the basis of the recovered areas, is universally applicable and a convenient method of eliminating the need for accurate injection of small quantities of lipid dissolved in highly volatile organic solvents. Reliance upon GLC for the quantification of the components separated by TLC allows much more

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sensitive and specific measurement than the charring by sulphuric acid, or determination of organic phosphorus, as frequently used for the estimation of neutral and phospholipids[4]. The possibility of regular cross-checks of the separated and recovered materials as well as reference comparisons to the original materials, permits the achievement of an accuracy not previously realized in the quantitative analysis of any mixtures of natural compounds of comparable complexity. With the increasing number of cross-checks, however, the amount of paper work increases tremendously and the analysis of several hundred samples may require considerable time for calculations. The summations of products and normalizations, however, are rather simple and lend readily to computer programming. By means of an adequately prepared program the data can be taken directly from the chart paper or print out integrator, entered on the computer cards and the concentrations obtained within a few minutes.

3. Speed of analysis

With the final calculations and normalizations handled by a computer, and the peak areas obtained by electronic print out integrators, the only time consuming steps remaining in this analytical system are the preparation of the original lipid extract and the transesterification following TLC or preparative GLC separations. Both TLC and GLC require about 1/2 to 1 h for each run, but several runs may be made at a time if enough instruments are available. The total analysis time for most lipid mixtures can therefore be reduced to a few hours and an overnight transesterification. In contrast, the time required to complete the separation of the neutral and phospholipid components by conventional analytical adsorption columns may require one or more weeks, and are unlikely to result in resolutions of comparable quality. Despite the great speed-up in the total analysis time already achieved, there is good reason to believe that further savings in time and labour are possible. The simplicity and general applicability of the integrated system suggests that at least certain stages of the system could be adapted to automation. At the moment the most obvious short-cuts are to be made in the employment of print out integrators for the measurement of the peak areas and the use of a computer for the final quantification, normalization and the expression of the analytical data in a meaningful form.

(*b*) *Disadvantages of the integrated systems*

In principle there seems to be no reason why the lipids separated by one TLC system could not be rechromatographed in another TLC system or be used for further gas chromatographic separation after elution as such or following transmethylation. A close examination of the experimental conditions commonly employed in lipid separations by TLC and GLC, however, suggests that certain precautions must be observed if true and reproducible results are to be obtained.

1. Oxidation on TLC plates

One obvious disadvantage might be the increased opportunity for oxidation of the unsaturated fatty acids due to the larger surface of lipid exposed to air in the TLC analysis. It has been shown[3], however, that under normal conditions of operation no detectable oxidation occurs within 10 minutes of the separation. Oxidation can be

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easily demonstrated by means of two-dimensional TLC. If highly unsaturated lipids are chromatographed in two dimensions in the same solvent system, the resolved substances line up diagonally if oxidation is absent. If the plate, after a run in one direction, is exposed to air for prolonged periods of time, polar oxidation products, which migrate more slowly, may be observed. OETTE[14] has described other TLC methods for the identification of lipid peroxides. It has been noted that auto-oxidation occurs rapidly if the chromatoplate is not protected by a solvent film[4]. It is therefore important that the silica gel sections containing the separated components be scraped off the plate while the layer is still wet and the scrapings be transferred directly into the eluting or transmethylating solution. In several studies[4,9,11,15,17] evidence has been obtained that indicates that the analytical plates can be processed rapidly enough to avoid the oxidation of polyunsaturated acids even when several lipid bands have to be located and scraped off. Replicate analyses for linoleic and arachidonic acids have given values comparable to those obtained after column separation[9]. The GLC analyses of polyunsaturated fatty acids obtained from lipids previously recovered from TLC plates have given results in reasonable agreement with values found by alkali isomerization for non-conjugated dienes, trienes and tetraenes[16,17].

Some changes from exposure to air must be expected with the more labile phospholipids such as phosphatidyl ethanolamine and phosphatidyl serine, if repeated separations by TLC are used for their isolation, unless special precaution is taken of operating largely under an inert atmosphere. It is possible that some of the minor unidentified GLC peaks commonly observed in fatty acid elutions represent decomposition products from the auto-oxidation of unsaturated fatty acids. It would appear, however, that the rapidity of the separation and the simplicity of sample preparation would recommend TLC especially for the handling of labile compounds since the time in which oxidation can occur is limited.

2. Adsorption on TLC plates

As a result of the application of TLC to the separation of various substances it has become increasingly apparent that TLC is most efficient when conducted under conditions approaching partition chromatography. In designing or selecting systems for quantitative TLC, therefore, attempts should be made to eliminate strong adsorption properties in the silica gel in order to eliminate the spreading and tailing of spots or bands. It has been observed[18] that with the ordinary silica gel preparations containing $CaSO_4$ as a binder, only acidic substances including fatty acids, sulpholipids, and the acidic phospholipids show appreciable adsorption characteristics. Much of this adsorption can be avoided by substituting the $CaSO_4$ with magnesium silicate, which attaches the adsorbent to the glass plate but shows minimal adsorption effects itself. Other workers recommend adding ammonium sulphate[19] and sodium acetate or sodium carbonate[20] to silica gel G to suppress adsorption effects. To completely eliminate the spreading, it is necessary to incorporate an acid into the chromatographic system, which prevents adsorption by depressing the dissociation of the acidic lipids.

It is obvious that badly tailing spots or bands are more likely to overlap with neighboring components and result in mutual contamination than well rounded spots or compact bands. Strongly adsorbed components are also difficult to recover from the adsorbent layers following the development of the chromatogram. This is especially true for the phospholipids the recoveries of which, as judged by extractable organic

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phosphorus, may frequently be less than quantitative. Thus phosphatidyl serine has been recovered after one- and two-dimensional TLC in yields of only 87 and 84 % respectively[21]. Conversion of the phospholipids into the less polar fatty acid esters by treating the silica gel with methanolic sulfuric acid results in complete recovery of the fatty acid parts[9,22]. Furthermore, strong adsorption is more likely to bring about degradation of the more labile polar lipids. Thus, highly active anhydrous silicic acid columns have a tendency to degrade cardiolipin[23], phosphatidyl ethanolamine[24] and the plasmalogens[25], while the silica gel layers developed with water containing solvent systems show no such adverse effects[26]. Efforts should therefore be made to avoid adsorption as much as possible and silica gel preparations should be tested for recoveries of lipids before they are used for quantitative studies.

3. Incomplete recoveries from GLC

The successful use of GLC for the quantification of the lipids separated by TLC depends upon the proper operation of the GLC systems, and a quantitative recovery of all components. Although it is commonly accepted that GLC allows quantitative fatty acid analysis, true quantifications have seldom been made. Usually the total amount of the lipid is ascertained gravimetrically and only the proportions of the major fatty acids are determined by GLC. Furthermore, the isothermal systems commonly employed in fatty acid analyses[27] are not very well suited for the determination of the great variety of acids found in a natural lipid mixture. While the medium chain length saturated fatty acids are effectively estimated in such systems, the recoveries of long chain unsaturated acids are low and difficult to evaluate because of the extremely long retention times and flat peaks. Low recoveries of long chain acids are also obtained on heavily loaded GLC columns under temperature programming. For an effective quantification of both short and long chain fatty acids, thin film columns combined with temperature programming give the best results and the sensitivity and reliability of the estimate can be greatly improved by the use of dual column systems. The choice of the GLC system and the available alternatives are discussed elsewhere in the text. It suffices to say that quantification of all fatty acids by GLC is theoretically and practically feasible, although not all commonly employed methods are satisfactory.

Suitable thin film columns have also been described for the GLC analysis of triglycerides[6] and steryl esters[7]. Although the recoveries of these components (average relative error 5 %) are somewhat less quantitative than those realized with the fatty acid methyl esters under optimum conditions (relative error less than 1 %), they compare favorably with any other methods of measuring the lipids resolved by TLC. With increasing molecular weight, the GLC recoveries of steryl esters and neutral glycerides become progressively lower and losses of up to 25 % have been encountered for compounds of the molecular weight of trierucin[28]. Low recoveries on GLC are also experienced for trilinolein and other polyunsaturated triglycerides although triolein and cholesteryl linoleate and arachidonate can be quantitatively recovered[7].

Phospholipids as such cannot be recovered from GLC columns, but their diglyceride parts can be analyzed readily in the form of their acetates[13] prepared by acetolysis. Because of the rather low molecular weight, the diglyceride acetates can be recovered quantitatively even when long chain unsaturated fatty acids are present. It is therefore seen that for an effective quantification of the lipid components resolved by TLC,

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a judicious choice of the appropriate GLC system is necessary. Although most components can be estimated on the basis of their fatty acid content, a direct quantification of the compounds, particularly after a further GLC resolution of the lipid class, provides much additional information. The need to use an internal standard for frequent checks of the recoveries, and as a general means of quantification, is not an additional burden but sound analytical practice.

3. PRACTICAL CONSIDERATIONS

During the past few years both TLC and GLC have become established as routine methods in the lipid research laboratory. Detailed accounts of the separations obtained and the general techniques to be used have been presented for both the TLC[3,4,10] and GLC[5,6,29], for the lipids under consideration. For a successful combination of TLC and GLC techniques for quantitative lipid analysis, however, only a few of the general systems have proved satisfactory and have provided superior data. Many others have caused loss or destruction of valuable samples with resultant disappointment. The following section reviews those modifications of the general techniques which have proved best suited to a mutual integration of the two techniques for rapid quantitative analysis of natural lipid mixtures. The discussion is preceded by a brief note on the choice of the lipid sources and the preparation of the extracts.

(a) *Preparation of lipid samples*

The availability of efficient TLC and GLC equipment has allowed an intensive study of the composition of natural lipid mixtures. Most investigators, however, have concentrated their efforts on a thorough analysis of the fatty acids of one or a few lipid classes from one tissue, and have made comparisons between the findings in health and disease. Rarely, however, can these data be generalized or even satisfactorily reproduced as the source of the lipids has not been adequately established or defined. Furthermore, in view of the work of GALANOS AND KAPOULAS[30] there may need to be a thorough re-examination of the methods of preparation of the original lipid extracts

1. Definition of source

In order to establish, with as much detail and accuracy as possible, the lipid patterns of normal and diseased individuals of both sexes and of different ages and nutritional status, it is undesirable to pool samples from different subjects. The analytical systems discussed here require only minute amounts of material and it is possible to determine the lipid composition of any part of even a small individual organ. As the analyses become more refined there is a need for still better definition of the origin of the lipid mixture and simple total tissue extracts may not be satisfactory. The lipid extractions must be preceded by tissue fractionation, isolation of well defined cellular fragments and their constituent lipoproteins or glycolipids, before a meaningful lipid analysis can be profitably attempted. Although a variety of subcellular fragments can readily be prepared and the lipid composition determined[26], only one attempt[31] appears to have thus far been made to ascertain the lipid composition of the lipoprotein complexes of individual subcellular organelles. Before individual lipoproteins can be effectively analyzed further improvements will have to be awaited in the fractionation

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of lipoproteins. Separations based on centrifugations in density gradients would not appear to bring about complete resolution of all lipoproteins and may not even reveal the true heterogeneity of the sample. Electrophoretic techniques have been shown[32] to be capable of much finer differentiation, but thus far have had only limited application in the isolation of lipoproteins for detailed lipid analyses.

2. Methods of extraction

Since the most complete analytical scheme cannot recover the information about fractions lost in the preparation of the sample, the methods used in the extraction and isolation of the lipid mixtures are as important as any of the separations or quantifications subsequently performed. For this reason much time and effort has been expended in devising the best system for each isolation. The general methods suitable for the preparation of lipid samples for TLC have been discussed by MANGOLD[3]. The lipid extraction procedures must give complete isolations and must not produce artifacts of esterification or oxidation. The possibility of formation of methyl esters of fatty acids during extraction of tissue lipids with chloroform–methanol has been demonstrated by LOUGH *et al.*[33], who reported that bicarbonate played a catalytic role in the formation of methyl esters of fatty acids. This observation has been confirmed by SKIPSKI *et al.*[34] who showed that the formation of the fatty acid methyl esters in serum lipid extracts could be prevented if the serum was subjected to dialysis against distilled water prior to the chloroform–methanol extraction. Formation of methyl esters of fatty acids was also prevented when non-alcoholic solutions were used for serum extraction without prior dialysis. The presence of methyl esters in other human and animal tissues, however, has been reported using chloroform–propanol and ether[35] and petroleum ether[36] as well as chloroform, acetone–chloroform, glycerol–chloroform and propylene glycol–chloroform[37] as the extraction solvents and therefore could not be the artifacts of sample preparation. When the Bloor mixture is used, complete extraction of lipids from very fatty tissues requires slight heating of the homogenate with ethanol–diethyl ether. Brain lipids are only partially extracted by this method[3]. Extraction of lipids according to FOLCH *et al.*[38] gives very good yields of complex lipids, such as proteolipids and gangliosides in a form suitable for TLC. Total lipid extracts prepared under these conditions usually also contain free amino acids and peptides, sugars, and other hydrophilic natural products which are carried into the extract through the action of lecithin and other solubilizers. It is very difficult to remove these contaminants completely unless some chromatographic system is used. The cellulose column methods are the most suitable for separating lipids from non-lipids. These are partition columns, however, and separations are performed by chloroform–alcohol–water mixtures which tend to produce channelling and often results in admixture of fractions[18]. The low solubility of triglycerides and steryl esters of saturated fatty acids in the water saturated systems leads to tailing into the water soluble fraction unless very large elution volumes are used, which is expensive and time consuming. Traces of sulphatide and phosphatidyl serine may be present in the water soluble fraction[18]. The suitability of other column systems for the purification of lipid extracts and a preliminary resolution of the more polar lipid classes prior to quantitative thin-layer chromatography has been reviewed in detail by ROUSER *et al.*[39]. However, in the preparation of lipid extracts satisfactory for quantitative TLC, using GLC with internal standards for the final quantification, the presence of trace

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amounts of non-lipids in the sample is not critical. In fact it may be desirable to reduce the amount of handling of the total lipid extract to avoid losses and possible oxidation. Since the extracted mixture is not to be weighed or examined by charring or colorimetry at any stage, there can be no interference from impurities and there is no need to purify the lipid extract to the same extent as would be required if the material was to be analyzed by conventional methods or by charring with sulphuric acid on the TLC plates.

Furthermore, it may be necessary to discontinue the practice of effecting a preliminary resolution of neutral lipids and phospholipids by adsorption column chromatography prior to the lipid class separations by TLC. In view of the demonstrated[25,26] degradative effects of silicic acid columns upon the phospholipids, the lipid fractions recovered from anhydrous adsorbent columns are unreliable for detailed quantitative work. There may however, be good reasons[9,19] to retain the DEAE cellulose column as an effective means of preliminary resolution of polar lipid classes for subsequent examination by the combined TLC–GLC approach.

The lack of interference of non-lipid constituents with the TLC separation and subsequent GLC quantification is best illustrated by the success of TLC following direct application of serum to thin-layer plates[40,41]. Thus, when 20 μl per spot of serum were applied to layers of 0.25 mm thickness and the plates developed three times to a height of 5–6 cm with chloroform–methanol (2:1, v/v) prior to the final development in the solvent system hexane–diethyl ether–acetic acid (80:20:1.5, v/v/v) the resulting chromatograms were virtually indistinguishable from those obtained when lipid extracts of the sera were chromatographed[41].

In connection with the selection of solvent systems suitable for the extraction of native lipid mixtures, the studies of GALANOS AND KAPOULAS[30] are of great interest. These workers have published results showing that natural phospholipids occur mainly as labile glycophospholipid and proteolipid complexes. These complexes are readily degraded, by the common extracting agents, to the types of phospholipids usually studied. Any one of these phospholipids, however, may have been derived from several different glycophospholipids. The modified extraction methods proposed by GALANOS AND KAPOULAS[30] apparently allow the isolation of the original lipid complexes and provide a new and possibly a biochemically more meaningful basis for the classification of the common phospholipids.

(*b*) *Quantitative TLC*

Successful use of TLC for quantitative work depends upon a clear recognition of the experimental variables involved and the need for their control at all times. In the present discussion only those aspects of the general technique of TLC are considered which are intimately related to an effective separation, recovery and subsequent measurement by GLC, of either the original lipid classes or their derivatives prepared by chemical modification of the separated components in the presence of the silica gel.

1. *Preparation of plates*

In the original combinations of TLC and GLC reported by GORDIS[42] and by BOWYER *et al.*[22] the thin-layer plates were prepared with silicic acid, which was ground and slurried with calcium sulphate in the laboratory. Most others have used commercial

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preparations of silica gel containing 5 to 13% calcium sulphate. Both types of adsorbent have yielded satisfactory plates for the separation and recovery of the common lipid classes. The presence of calcium sulphate in the gel was found[22] not to have any significant effect upon the recovery of the fatty acid methyl esters or the transmethylation process carried out in the presence of the scrapings. It has been shown, however, that the amount of the calcium sulphate present has an appreciable influence upon the rate of migration and the extent of separation of different compounds. Thus the R_F value of methyl oleate was seen[43] to increase linearly when comparisons were made using plates prepared with 0, 10 and 26% (w/w) calcium sulphate as binder. Other authors[20] have reported that the R_F values of phosphatidyl serine on silica gel G (Merck) plates, prepared with calcium sulphate, depend upon the amount of the phospholipid applied, and that this load effect can be eliminated by using "basic" silica gel G plates (silica gel slurried with a 0.01 M Na_2CO_3 in water). Such plates were also observed to give better resolution of phosphatidyl serine and phosphatidyl ethanolamine as well as phosphatidyl inositol along with other phospholipids. Excellent separations of all phospholipids including the cephalins have been obtained by PARKER AND PETERSON[44] on a specially washed silica gel H (Merck), which is adhesive without gypsum additive. Improved separations of neutral lipid mixtures have been obtained on silica gel G containing no binder; by developing the chromatoplate in two steps by two solvent mixtures of different polarities[34]. The substitution of magnesium silicate for calcium sulphate has been shown[18] to result in less tailing and more compact spots for the more acidic substances including free fatty acids, sulpholipids, and the acidic phospholipids. The magnesium silicate firmly attaches the adsorbent to the glass plates, shows minimal adsorption effects and does not interfere with the transesterification of the fatty acid esters when present in the reaction mixture[9]. The separated components give compact spots and may be developed in a second direction (two-dimensional TLC) without significant loss of material.

The incorporation of silver nitrate into the adsorbent powder has an effect upon the R_F value opposite to that of calcium sulphate. With increasing concentration of silver ions, the R_F values decrease[43]. Usually silica gel G containing 13% calcium sulphate as binder is used and the silver nitrate is incorporated into the slurry at a level of 6–25%[10]. Spraying of prepared plates with solutions of silver nitrate to produce even wetting is an unsatisfactory method of obtaining uniform impregnation with silver nitrate for quantitative work. For the separation of natural lecithins, ARVIDSON[15] has successfully employed gypsum-free silica gel H impregnated with silver nitrate at the 30% level. Silica gel G with 13% gypsum was satisfactory for the fractionation of the more unsaturated lecithins, but the resolution of monoenes and dienes was poor. This was apparently due to the presence of the calcium sulphate, since silica gel H to which a comparable amount of gypsum was added also gave the same results as silica gel G.

In addition to the concentration of the binder and the additives modifying the properties of the silica gel, the migration rate and final separation of certain lipid components is very sensitive to the water content of the adsorbent layer. Thus on thin layers containing less water there is a greater migration of the more acidic lipids when compared to the thicker layers where there remains relatively more water following equal periods of activation. The higher water content apparently results in a

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somewhat greater binding of the acidic lipid to the stationary phase[18]. The neutral lipids on the other hand are little affected by minor variations in the water content of the plate. This characteristic behaviour of the acidic and non-acidic lipids in response to differences in the water content can be exploited in the design of solvent systems to obtain optimum spacing of lipids for quantitative separation by utilizing adsorbent layers of different thicknesses. With further increases in the water concentration, the effect is reversed and all lipids are more readily eluted or displaced. This phenomenon may be utilized, in avoiding adsorption of the more acidic lipids at the origin by wetting the area of application, and for obtaining improved mixtures of eluting solutions for the recovery of the lipids from the TLC plates. The spreading of the cardiolipin spots on silica gel G plates containing calcium sulphate may be prevented by preparing the slurry with 0.01 of 0.02 *M* sodium chloride instead of water[18]. The addition of 0.5 % methanol to the slurry mixture for chromatoplate preparations has been claimed[45] to reduce the surface tension and produce a more even spreading of the silica gel.

Plates of 20 × 20 cm size and a 250 micron thickness of adsorbent (with or without binder) have been found most advantageous in the separation of lipids for subsequent GLC. These are readily prepared with most applicators and provide sufficient amounts of major and minor components when the material is applied as a band. For ultramicro analyses, a 20 × 20 cm plate has been used for a preliminary two-dimensional chromatography as the material present in a single spot was sufficient for GLC[9]. Plates thicker than 250 microns increase the developing time and their use may result in less satisfactory resolution and overlapping of bands due to difficulty in preparing even layers and equilibrating them. The Desaga (C. Desaga GmbH, Heidelberg, Germany) type of spreader has been most often used for the preparation of both ordinary and silver nitrate impregnated plates. After the plates have dried for about 1/2 to 1 h at room temperature, they are activated immediately before use by heating in an oven at about 110° for 1 to 2 hours. Those containing the silver nitrate may be activated similarly but for special purposes longer periods of time (5–24 h) and higher temperatures (150–180°) may be necessary[15]. The actual time and temperature used in any one laboratory depends on the type and efficiency of the heating oven.

In order to avoid contamination of the analyzed samples, the adsorbent powder and all reagents should be free of impurities soluble in the developing or the transesterifying solvents or extractants that may give rise to false peaks in the GLC. It is desirable to wash the activated plates prior to use, with chloroform or the solvent mixture subsequently to be used for the elution of the lipids[4]. By placing the plate in a developing tank and allowing the washing solvent to ascend to the top, most of the soluble material is carried with the solvent front and the adsorbent blanks reduced. After removing the plates from the tank, the solvent is allowed to evaporate at room temperature and the residual moisture is driven off during reactivation in an oven at 110° after which the plate is ready for use. A similar washing procedure can be used for cleaning up silver nitrate plates but the washing solvent should not contain a high proportion of aqueous phase which would wash out the silver nitrate. Because of the high photosensitivity of the silver salts, the silver nitrate plates should be prepared and used in the dark. According to ARVIDSON[15] no ill effects are noted when partially discolored silver nitrate plates are used for the separation of lecithins.

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2. Application of samples

It has been known for some time that the application of samples to the chromatoplates in a narrow band or streak rather than a spot promotes better separation[46]. The application of samples in the form of a band also allows the handling of larger quantities of material. The most reliable applications of bands for purposes of quantitative separations are obtained by means of mechanical applicators. The electromechanical applicator of Desaga (C. Desaga GmbH, Heidelberg, Germany) which allows an automatic sample application to TLC plates is of interest. A motor drive ensures constant speed traverse of the sprayer across the plate. Stops are provided to reverse the direction of travel without lag at the end of each traverse. The total volume of the sample holder is about 4 ml. A source of compressed gas (nitrogen) is required to discharge the sample solution. The rate of feed is controlled by altering the gas pressure.

The Chromatocharger of Camag (Camag, A.G., Muttenz, B.L., Switzerland) is a hand operated instrument that also allows the delivery of a solvent containing lipid solute in a controlled manner. Although intended primarily for preparative TLC on thick silica gel plates, both of these instruments are well suited for the application of samples to essentially analytical plates in a quantitative manner. Any desired amount of material may be applied in a solution of appropriate dilution.

For two-dimensional TLC or when only very small amounts of material are available, the sample is applied as a small spot (up to 1 cm in diameter) by means of a 10 μl Hamilton (Hamilton Company, Whittier, California) syringe.

The amount of the lipid applied to the plate varies with the complexity of the mixture and the thickness of the adsorbent layer. It has been found[3] that the application of 100 to 150 μg of total lipid extract permits the detection of all compounds present in the mixture in quantities of 1% or less (usually as little as 0.3 μg can be detected). In work with human plasma samples[47] it has been found convenient to prepare two 20 × 20 cm plates from the total lipid extract obtained from 1–2 ml of plasma. The amounts of the individual lipids isolated are sufficient for the determination of the fatty acid composition of each band as well as for a direct GLC of part of each of the original lipid classes. There is usually sufficient material for several injections into the GLC of each ester mixture and the samples may be handled in commonly available screw cap vials (9 ml capacity) with conical polyethylene or Teflon inserts. The application of samples to thin-layer plates under nitrogen prevents oxidation and decreases the influence of atmospheric humidity on R_F values. A special box has been described[48] for this purpose and its use is essential when samples are banded by hand.

3. Development of plates

The solvent systems chosen for the development of the chromatoplates vary with the particular lipid mixture present and the separations required. Generally two solvent systems are used. One separates the neutral lipids and leaves the phospholipids at the origin, the other separates the phospholipids and carries the neutral lipids with the solvent front. Several different solvent systems can be used to obtain this effect. A popular and quite satisfactory neutral lipid system[3] consists of petroleum–diethyl ether–acetic acid in a ratio of 90:10:1 (v/v/v). SKIPSKI *et al.*[34] recommend that for optimum resolution the neutral plates be developed in two steps by two solvent mixtures of different polarity. In this stepwise development the solvents are essentially

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a combination of the two systems previously used by KAUFMANN AND MAKUS[49] and MANGOLD AND TUNA[50]. SKIPSKI *et al.*[34] report that in over 100 experiments the application of this TLC system to the separation of neutral lipids extracted from human serum and rat liver proved to be simple, versatile, reproducible and reliable. In the author's laboratory several hundred samples of human plasma[47] and dog lymph[51] have been analyzed for neutral lipids by TLC using the single solvent system heptane–isopropyl ether–acetic acid (60:40:2, v/v/v). Both systems have allowed the separation of total lipid extracts into individual lipid classes in order of increasing polarity: hydrocarbons, steryl esters, methyl esters, triglycerides, free fatty acids, cholesterol, diglycerides, monoglycerides and phospholipids. The partial overlapping of cholesterol and diglycerides, and of the triglycerides and the methyl esters of fatty acids is not serious as the cross-contamination can readily be ascertained by GLC and appropriate corrections made.

In the original proposal of BOWYER *et al.*[22] the phospholipids were fractionated by a mixture of chloroform–methanol–water in a ratio of 80:25:3 (v/v/v). Under these conditions the neutral lipids migrate just below the solvent front and are followed by the cephalins (not completely resolved), lecithin, sphingomyelin and lysolecithin as distinct bands. The solvent system of SKIPSKI *et al.*[52], consisting of chloroform–methanol–acetic acid–water (25:15:4:2, v/v/v/v) allows a complete separation of phosphatidyl serine and phosphatidyl ethanolamine as well as phosphatidyl inositol from each other and the rest of the phospholipids. The separation can be carried out on the whole lipid extract with the neutral lipids migrating to the top. In case of lipid mixtures containing cardiolipin, it may be necessary to remove first the neutral lipids either on a suitable column[53] or on a separate TLC plate[54], as the fast moving cardiolipin tends to run into the neutral lipid band. PARKER AND PETERSON[44] have reported that with a multicomponent system such as that described by SKIPSKI *et al.*[52], reproducible separations appear to be dependent upon complete saturation of the atmosphere in the chromatographic tank. This condition is conveniently met when a saturation chamber of small volume is used[14]. ARVIDSON[15], on the other hand, has claimed that the best separations of lecithins on silver nitrate impregnated plates are obtained in non-equilibrated chromatographic tanks. To avoid peroxidation of the lipid samples during development, the developing solutions should be deoxygenated and the atmosphere of the developing chamber inert.

4. Detection of lipids

Prior to the recovery of substances from the chromatoplate, the positions of the separated components must be located. A host of reagents and techniques have been used[3,4,10] for this purpose and a comprehensive discussion of them is beyond the scope of this report. There are certain general guides, however, which should be followed if the recovered substances are to be used for subsequent quantification by GLC. The technique must be sensitive and universally applicable. Such general methods of locating organic compounds on inorganic TLC plates as charring with sulphuric acid must be avoided or a pilot plate be prepared under identical conditions[4].

Almost every lipid can be recognized after spraying the plate with an alcoholic solution of 2,7-dichlorofluorescein[3]. On examining the plate under U.V. light (Mineralight, Ultra-Violet Products Inc., San Gabriel, California) the lipids appear as light green fluorescent spots on a dark background. With this reagent, or its

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dibromo equivalent, it is possible to detect as little as 1–5 $\mu g/cm^2$ of a compound. Spraying with an alcoholic solution of Rhodamine B also can be used to locate lipids on TLC plates prior to elution or transmethylation. As little as 1 $\mu g/cm^2$ of lipid has been detected by examining the plate under U.V. light, as the lipids then appear as dark violet spots or bands on a pink background[3]. Rhodamine 6G can be used in aqueous solution and the plates viewed under U.V. while they are still wet[9]. Keeping the plates moist may prevent oxidation but the water has to be removed before transmethylation can be successfully completed. Bromothymol blue (0.1 %) dissolved in 10 % alcohol in water and made basic with ammonium hydroxide is a good indicator for the location of phospholipids and related compounds because it is stable and easy to remove, and is a sensitive indicator on wet plates[55]. It is good practice to use the aqueous dyes for the location of those bands which are to be extracted for direct GLC examination as it combines instantaneous deactivation of plates with the location of bands. Usually, however, it is convenient to locate both, phospholipids and neutral lipids, by spraying with one or the other of the fluorescent dyes.

The fluorescent indicators are most sensitive when the plates are dry or nearly dry and they must be viewed under ultraviolet light which further increases the chances for changes in light sensitive compounds such as phospholipids. If the phospholipids are to be removed intact, the chromatoplates should not be allowed to dry completely at any of the steps of the analytical procedure, and all solutions used in the course of the recovery of these compounds should be protected from atmospheric oxygen by an inert atmosphere (nitrogen).

Impurities in the fluorescein and other dyes should be removed by recrystallization or chromatography as these have been observed after petroleum extraction as peaks on GLC. Thus, GLC of the fluorescein sprayed blank areas of adsorbent, of size comparable to those of the reference phospholipids have been reported[44] to give a peak with the same retention time as methyl palmitate, when 12 % ethylene glycol succinate polyester is used as the liquid phase. The extent of interference can be greatly minimized by using as dilute solutions of the dyes as possible and by spraying only sufficiently to make the bands clearly visible. Spraying with 0.001–0.002 % solutions of these dyes in alcohol or water usually allows a reliable location of all bands and an effective GLC following transmethylation without a prior removal of the fluorescent spray reagents.

Iodine will stain all unsaturated lipids and some nitrogenous saturated lipids brown on a light yellow or white background[3]. It is very sensitive and will detect as little as 1 $\mu g/cm^2$ of a monounsaturated compound when the plate is exposed to iodine vapor for a few minutes[3]. Most saturated lipids, however, become only faintly colored even when present in high concentrations. BELFRAGE *et al.*[56] have used iodine staining to detect the location of phospholipids on TLC plates by lightly spraying the plate with 1.5 % (w/v) iodine in methanol. Others[21] have limited the exposure to iodine vapor to 30 seconds. The brown spots disappear in a few minutes when the plate is removed from the iodine tank but unfortunately it does not necessarily mean that all the iodine has been dissociated from the compounds. In view of the possible iodination of the double bonds[57] or an irreversible attachment of the iodine to the nitrogens, it is preferable not to expose the lipids to iodine. A partial loss of the unsaturated fatty acids on GLC or an incomplete recovery of the phospholipids on

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elution from the TLC plate or rechromatography appear to be the most obvious consequences from this type of band detection. Staining with iodine, however, may be adequate for the location of phospholipid bands which are to be used for the determination of organic phosphorus[44] or radioactivity[45] in the presence of the scrapings from the plate. Iodine is of little use for the visualization of compounds separated on silver nitrate impregnated plates[10].

Many compounds, including phospholipids and sterols, may be made visible long enough to be detected by spraying the plate with a neutral solvent and viewing it with transmitted light[4]. A technique permiting the determinating of a distribution pattern of lipids over the entire plate, and which may be very useful for the determination of minor components as well as components not well separated from each other, has been described by SNYDER[58]. It includes an automatic scraper which recovers zones of adsorbent, of predetermined width, directly into vials for analysis of radioactive lipids by scintillation counting. It could possibly serve as part of the assembly line in an automated TLC–GLC analytical system.

5. Recovery of lipids

Intact lipids may be recovered from the TLC plates by scraping off all deposit from appropriate areas of the chromatoplate into a suitable eluting solution. The adsorbent is readily dislodged by a razor blade or a sharp Teflon spatula. The use of miniature vacuum cleaners[59,60] would appear to expose the lipid sample to an unnecessary aeration. Care must be taken in the choice of solvents and extraction technique to insure absence of alteration and complete recovery of substances. The phospholipids are particularly unstable and require rapid processing. According to PRIVETT *et al.*[4], natural lecithins cannot be stored for prolonged periods without some alteration even at low temperatures. The deterioration of these compounds appears to take place regardless of the degree of unsaturation because even fully saturated lecithins isolated from natural sources tend to change. Deterioration of the phospholipids usually may be detected by the formation of products that have different R_F values on TLC than the parent compounds. A satisfactory method for storing phospholipids for short periods of time is one at low temperature in the presence of air-free solvent. It should be noted that the fatty acids of the phospholipids may be destroyed to some extent also during such commonly applied reactions as interesterification or enzymatic hydrolysis. PRIVETT *et al.*[4] have obtained evidence that these compounds are converted to some extent into hydrocarbons.

Several procedures have been described for the quantitative recovery of intact phospholipids from TLC plates[4,15,17]. According to PRIVETT *et al.*[4] the locations of the compounds are marked with a needle and the silica gel is scraped into beakers containing about 20 ml of chloroform–methanol–water (65:40:5, v/v/v). Each area from which the adsorbent is removed is then swabbed with a small piece of cotton saturated with the extraction solvent to insure complete recovery of the sample. The cotton, which is pre-extracted with aqueous as well as organic solvents, is washed with a small amount of chloroform and handled with forceps at all times. The slurry of each component is transferred to a sintered glass funnel and washed with several small portions of the same mixture of solvents. The filtrates of each slurry are combined and evaporated to near dryness. The residues are dissolved in small volumes of chloroform and any insoluble matter removed by filtration through a small cotton plug inserted in the

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syringe used to make the transfer of the solutions to another flask. This solution is evaporated to near dryness and diluted to volume for further analysis. PRIVETT *et al.*[4] also describe in detail a modified procedure which is followed if it is necessary to remove the indicator dye prior to analysis. Although filtration is the usual practice in these isolations, the washing and extraction techniques may be carried out by centrifugation.

Quantitative recoveries of polyunsaturated fatty acids or esters and phospholipids may also be obtained by the same general method from adsorbents impregnated with silver nitrate, except that a small amount of hydrochloric acid should be added to the solution to insure complete breaking up of the silver–fat complexes. Simple esters of fatty acids are recovered by a final extraction into petroleum ether which is washed to remove the acid and silver salts. The phospholipids are recovered into chloroform solution after washing with water to remove acid and silver salts. Centrifugation in micro-separatory funnels promotes the separation into phases and reduces losses due to emulsion formation.

Alternatively, descending TLC may be used in the recovery of phospholipids to eliminate deterioration. The disadvantage of this method is the relatively long time required to elute all the samples from the plate. However, by comparison it has been shown[4] that the changes in the phospholipids during ascending TLC occur generally with the handling of the sample after development of the plate. By continuous application of the solvent to the plate, all of the substances can be eluted without any evidence of structural change.

When further TLC or GLC subfractionation of the individual lipid classes is not required, the conditions for the recovery of the lipids from the chromatoplates may be greatly simplified. Since for subsequent GLC analysis of the fatty acids, methyl esters are required, one is concerned only with a quantitative recovery of the fatty acid part of the molecule. The elution solvents can therefore be so chosen as to permit a complete displacement of the lipids from the adsorbent, and a complete transmethylation of the esters or a methylation of the free acids, simultaneously. In such a case the silica gel scrapings are transferred to a suitable container, dried, if necessary, and covered with the transmethylation mixture, usually a solution of sulfuric acid (10%, w/v) in anhydrous methanol. The containers are closed with a Teflon lined screw cap or sealed with flame and heated at 80–110° overnight (8–16 h) to complete the conversion into the fatty acid methyl esters. Special re-usable vials have been designed for this purpose[61]. The methyl esters are extracted into hexane and after addition of a suitable amount of internal standard are quantitatively analyzed by GLC. In the absence of adsorbent the transesterifications may be carried out at 70°[9]. Flushing the tubes with nitrogen prior to closing and the addition of a crystal of hydroquinone[22] or 0.02 % of α-tocopherol[62] minimizes oxidation and does not interfere with analysis.

The diglyceride phospholipids may be recovered from the TLC plates in the form of their diglyceride acetates, following acetolysis in the presence of the silica gel[13]. The conditions of acetolysis are similar to those described by RENKONEN[11] except that there is no need for a prior elution of the phospholipids from the adsorbent. This results in a considerable saving of time and possibly in a higher yield of the lipid. The diglyceride acetates are recovered after equilibration of the reaction mixture with chloroform–methanol–water (80:40:30, v/v/v) and filtration. The acetates may then

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be subjected to chromatography on silica gel impregnated with silver nitrate as previously described[11] and the individual lipid classes recovered in the usual way and transmethylated, or the transmethylation may be carried out directly in the presence of both the adsorbent and the silver nitrate.

(c) *Quantitative GLC*

The separation and quantitative measurement of fatty acids was one of the first objectives of gas–liquid chromatography[63]. The subsequent rapid development of the technique and its application to other lipids, however, has changed the emphasis from the aspects of quantification to give priority to qualitative separations and the assessment of the proportional recovery of the peaks. The measurement of the absolute lipid concentration in a given sample therefore has seldom been made. This apparently has been due to the lack of accurate and convenient methods for the introduction of exact amounts of fatty materials into the gas chromatograph and lack of confidence in methods involving the use of the internal standard. Only when gravimetric, colorimetric and specific analytical methods have been impractical have quantitative analyses of the total lipid samples been made by means of internal standards. Although the elegance of using some foreign fatty acid methyl ester as an internal standard in the GLC of fatty acids has been expounded[61–66] and sample applications in the study of the composition of total lipid extracts[47,51], triglycerides[67,68] and phospholipids[54,69] illustrated, all too frequently the analysts have resorted to some colorimetric technique (total glycerol or total phosphorus) for the estimation of the overall concentration of a specific lipid class. It appears to have escaped notice that the fatty acid part of these lipid esters provides the bulk of atoms of the triglyceride and phospholipid molecules and that these carbon rich components can be measured to a much greater degree of precision and sensitivity than the organic phosphorus or glycerol residues which account for only a small part of the total molecule. Furthermore, the estimation of the phospholipid concentration in the TLC fractions on the basis of the fatty acid concentration is not only based on a sound analytical approach but it also offers advantages experimentally. The combination of the transesterification and the elution steps allows the use of much stronger elution solvents for the displacement of the less strongly adsorbed methyl esters, resulting in a more complete recovery of all components from the TLC plate. In contrast to this reliable preparation and recovery of the fatty acid esters, is the troublesome elution of the phospholipids[21] and the less than adequate determination of organic phosphorus in the presence of silicic acid[44]. Finally, the use of the fatty acid ester values for the quantification of the original lipid concentrations, provides additional information regarding the concentration of each acid in the particular lipid class, in the same step, resulting in economy of time and fewer manipulations. Though precedent may dictate that organic phosphorus determinations be used as the basis for calculation of phospholipids when working with sufficiently high lipid concentrations, there is no reason why attempts to measure organic phosphorus should be made when attempting to determine phospholipids in the nanogram range. Appropriate TLC of these lipids (two dimensional TLC if necessary) provides the required degree of selectivity and specificity. In view of these considerations there would appear to be no reason why the GLC analysis of the fatty acid composition should not be accepted also as an adequate measurement of the concentrations of

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all lipid classes, when sufficient material is available for conventional total lipid determinations. The involved calculations based on the use of the internal standard can be greatly simplified and largely eliminated by the use of computer programs of general applicability. This system of lipid analysis is widely applicable, simple and amenable to automation. Properly executed, it allows numerous cross-checks and back calculations providing complete analytical control and nearly absolute specificity in the final account.

In many cases the quantifications of the lipid classes separated on TLC plates can be readily performed by means of direct GLC. Thus, with the help of an internal standard it is possible to determine the concentration of the total triglyceride or steryl ester, as well as to estimate the proportions of these compounds contributed by specific molecular weights[6]. Following a TLC on silver nitrate impregnated plates, direct GLC of the resolved components can provide quantitative estimates of the various esters containing none, one, two or more double bonds per molecule. The values obtained for the esters in the various subclasses derived from TLC and/or GLC separation, can readily be related back to the concentrations of the orignal ester mixtures or to any one of a number of subfractions obtained in the course of the analytical fractionation. In fact a constant reference to the starting mixtures is absolutely necessary in order to avoid cumulative losses as explained elsewhere in the text, and to arrive at correct normalized ester or fatty acid values. The diglyceride acetates derived from phospholipids by acetolysis may be subjected to a similar GLC analysis, as can other properly prepared derivatives of monoglycerides and diglycerides.

1. Selection of GLC systems

Improved methods for quantitative GLC of fatty acids have been discussed by HORNING *et al.*[27] who also present analytical data obtained by interlaboratory comparisons of standard mixtures of fatty acid methyl esters. The analytical conditions described, and recommendations made, are satisfactory for the quantification of common fatty acids, such as those present in the glycerides of animal fats. For complete analysis of the fatty acids of many natural lipids, isothermal gas chromatographic techniques are inadequate. The short chain acid esters are lost in the solvent front while the long chain acid esters are recovered incompletely or not at all. In order to determine quantitatively all the fatty acids, thin films of suitable stationary phases and temperature programming are essential. Excellent recoveries, and for many purposes satisfactory resolutions of fatty acid esters, are obtained on 5% SE-30 columns. The low bleed rate of adequately conditioned silicone columns permits the scanning of the complete range of fatty acid esters from C_2 to C_{30}, and higher if necessary, by temperature programming, from ambient to 300° on single column instruments[47]. The silicone gum does not separate oleic and linoleic acid and these must be recorded as a combined percentage. Since a preliminary separation of the natural lipids within each lipid class, on the basis of unsaturation, by means of silver nitrate TLC, may have already been made, the oleic and linoleic acid esters are likely to have already been effectively segregated and no actual overlap may result. With regard to measurement there is no problem as it is based solely on the amounts of the acids of different molecular weight or chain length[47,70]. The silicone columns also permit a quantitative estimation of any hydroxy acids[71]. All the commonly encountered unsaturated fatty acids of medium and long chain length can be separated in a single run

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under conditions of temperature programming with 1% EGGS-X (Applied Science Laboratories, Inc., State College, Pennsylvania) as a stationary phase using a 12 ft. column[72]. This liquid phase is a copolymer of ethylene glycol, succinic acid and a dimethyl siloxane monomer and over the working range shows a low bleed rate, which can be reduced to minimum by the use of thin film columns. The methyl esters of nervonic and lignoceric acids on this column are separated with about the same degree of resolution as found for C_{16} and C_{18} monoene fatty acid methyl esters[73]. There is a slight rise in the base line when the column is programmed from about 120° to 215°, which can be eliminated by the use of dual column systems or suitable electronic base line drift correctors. The dimethyl acetals and fatty acid methyl esters derived from human serum cephalins have also been well resolved by such columns. Comparable separations of saturated and unsaturated fatty acid esters on temperature programming have been obtained[47] on thin coatings (3–5%) of another silicone polyester (ECNSS-S, Applied Science Laboratories, Inc.). Under isothermal conditions, FELDMAN AND ROUSER[74] were able to analyze quantitatively nanogram quantities of fatty acid methyl esters when a coating of 10% ECNSS-S on 100–120 mesh Gas Chrom P was used. The preparations of the silicone polyester copolymers, however, are variable and some batches give badly tailing runs with little or no resolution between the saturated and unsaturated fatty acids of the same chain length.

With the introduction of the double column systems[62,74] for the separation of fatty acid esters, it is now possible to obtain adequate temperature programmed runs also with the popular polyester columns, which for such purposes may be optimally made with a liquid phase concentration of about 6% of the weight of the support[54,62]. The desired separations of the saturated and unsaturated fatty acid methyl esters are maintained and the runs greatly shortened.

For establishing the amounts of the fatty acid methyl esters, a flame ionization detector is preferred. Results with an argon ionization detector have been found to be unsatisfactory, giving low values for the late components[72,73]. In order to obtain correct ionization responses for both early and late components in the hydrogen flame ionization detector during temperature programming, it is necessary to maintain constant flow rates through the detector. Under rigidly controlled conditions, GLC methods for fatty acid analyses give reproducible results with small error even with a thermal conductivity detector and agree with the stated composition of primary standards with a relative error of measurement of 1.5 to 3% at a 99% confidence level, suggesting a precision approaching that of conventional spectrophotometric measurements[75]. Under less than optimum conditions, relative errors of 5% may be encountered for most fatty acids[22]. Even then, the quantitative data derived from a combined TLC–GLC examination of a plasma lipid mixture gave as good results as a silicic acid column–GLC combination[22]. Erratic values can usually be avoided if the fatty acid peaks in the GLC recording are properly attenuated so as to produce reasonable deflection (50% of full scale) and width (proper recorder speed). In double column systems where a true base line compensation exists also during temperature programming, the accuracy of the area measurement of sharp peaks may be greatly improved by using electronic printout integrators (Infotronics Corporation, Houston, Texas).

Quantitative methods for the GLC analysis of high molecular weight esters of fatty acids, such as glycerides[6] and steryl esters[7], have thus far found only limited

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application. Short columns (18 in. × 1/8 in. O.D.) and thin films (1–3 %) of liquid phase are necessary. Such columns allow complete recoveries of steryl esters, including cholesteryl arachidonate, and triglycerides up to tristearin. Higher molecular weight esters, up to trierucin, are incompletely recovered and correction factors must be used for their quantification[28]. Of the available liquid phases only SE-30 (General Electric Co.) and JXR (Applied Science Laboratories, Inc.) have proved satisfactory[6,28] at the high operating temperatures (200–350°). The separations are based on the molecular weight of the compounds and there is no significant resolution of the saturated and unsaturated fatty acid esters of the same chain length. Some fractionation of the positional isomers of mixed long and short chain fatty acid esters of glycerol has been detected, but practical separations have been realized only with the α- and β-monoglyceride acetates[76] and butyrates[70].

Although no specific recommendations can be made with regard to the choice of the gas chromatographic equipment, some restrictions are clearly necessary if a reasonable precision (defined as repeating analysis on the same sample in different laboratories, by different analysts, using different equipment) is to be obtained in comparative studies. The problem of unsatisfactory precision frequently observed in interlaboratory comparisons[77] cannot be solved by an individual analyst in a specific laboratory who is obtaining satisfactory reproducibility. In the mean time the most rigorous specifications of conditions and procedures should be demanded with each compilation of data collected at the molecular level.

2. Preparation of derivatives

The methyl esters of fatty acids necessary for the GLC may be obtained by transmethylation of the lipids or by a methylation of the fatty acids recovered after saponification. Both techniques have been extensively employed and a variety of reagents described[5,29]. Special advantages have recently been claimed[78], for 5 % perchloric acid solutions in methanol as rapid methylating agents for free fatty acids at low temperatures. Because of the small quantities of lipid involved, the preparations of the methyl esters are best conducted by means of transmethylation. Saponifications and extractions of the acids inevitably result in losses due to foaming and the increased number of transfers. BOWYER *et al.*[22] found that all lipid fractions could be transmethylated, in the presence of silicic acid from the plate, by refluxing with 10 % (w/v) sulphuric acid in dry methanol for 1 h at 80°, except for sphingomyelin which should be refluxed for 16 h. A crystal of hydroquinone was added as antioxidant. Comparable recoveries were obtained from samples transmethylated directly and from samples esterified after recovery from silicic acid columns. FELDMAN AND ROUSER[9] have made similar observations regarding the effect of the silicic acid adsorbent, but felt that considerbly higher temperatures (110°) should be used for a complete transmethylation of nanogram quantities of sphingomyelin. They conducted all their transmethylations overnight with a similar solution of sulphuric acid in methanol (2 ml) but used sealed tubes and nitrogen atmosphere to contain the reaction mixture. On the following day the tubes were cooled, opened, and 1 ml of water was added. The esters were then extracted with several 0.5 ml portions of hexane and analyzed by GLC. The fatty acid mixtures recovered under these conditions, for a sample of pure cerebroside, compared favorably to those recovered from the same material without TLC and without silica gel in the transmethylating mixture.

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The use of methanolic sulphuric acid for the procedure is preferred in view of the report by JOHNSTON AND ROOTS[79] describing contamination from the use of methanolic hydrochloric acid as a methylating agent in the ultramicro analysis of methyl esters by GLC. Furthermore, the Special Task Group for Preparation of Methyl Esters has completed a draft of a Tentative Method for the preparation of methyl esters by the methanol–sulphuric acid procedure for submission to the Instrumental Techniques Committee with recommendation for referral to the Uniform Methods Committee for inclusion as a Tentative Method of the American Oil Chemists' Society[77]. Nevertheless, transmethylations by methanolic hydrochloric acid[42,61,80] as well as by methanol–BF_3[16,17] have given highly satisfactory results in a number of laboratories. LINDGREN *et al.*[81] have discussed sources of contamination other than methylating agents.

The discovery that transmethylations of lipids can be satisfactorily completed in the presence of silica gel[9,17,22,42] demonstrates that prior elution of the lipid as described by other investigators[50,59,73] is an unnecessary and time consuming step. In addition, direct interesterification of the adsorbed lipid diminishes the possibility of alteration (auto-oxidation) as a result of the extra handling involved in an elution procedure. There is no possibility of loss of any components during esterification in the presence of the silica gel, and the resulting fatty acid analysis appears to be a true reflection of the composition of the total lipid class.

For a further GLC subfractionation and quantification of individual phospholipid classes on the basis of molecular weight[13], it is necessary to convert the phospholipids into their diglyceride acetates. The experimental procedures for this purpose have been adopted from the original report of BEVAN *et al.*[82] and from the studies of RENKONEN[11]. It has been found[13] that the general conditions given by RENKONEN for the conversion of the phosphatidyl cholines, serines and ethanolamines into their diglyceride acetates are also satisfactory for the formation of these derivatives in the presence of the silica gel scrapings from the TLC plate. As explained for the *in situ* transmethylations of the fatty acids, such practice economizes in time and labour and frequently results in better recoveries of the lipids. In view of the growing popularity of the *in situ* transmethylation, the *in situ* acetolysis should prove to be an equally rewarding transformation for the study of the molecular populations of these important biological compounds. It has not yet been ascertained whether or not the acetolysis can also be effectively performed in the presence of silica gel impregnated with silver nitrate, which is used in the separation of intact lecithins on the basis of unsaturation. For further GLC examination of the monoglycerides, such as those recovered from pancreatic lipase digests, the silyl ethers are best suited[76]. Although simple diglycerides can be examined as such or following acetylation[6], there is reason to believe that isomerization occurs when an unsubstituted hydroxyl group is present during chromatography.

The transmethylation systems based on inorganic acid catalysis are unsuitable if both the fatty acid and the alcohol parts of the esters are to be quantitatively recovered[16]. Because of isomerization and partial dehydration of the alcohols under the anhydrous conditions, strong acids and high temperatures are to be avoided. In order to recover the alcohols also, the transesterifications are conducted in the presence of sodium methoxide. The fatty acid methyl ester and free cholesterol mixture, for example, resulting from the transmethylation of cholesteryl esters[83] can then be analyzed directly by GLC on SE-30 columns. The fatty acid esters are eluted first and the

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cholesterol last. The run can be conveniently completed in a minimum of time by means of temperature programming. Where both the acid and the alcohol parts are soluble in organic solvents, the esters may be saponified in the presence of the silica gel and the saponification products recovered by extraction. The fatty acids are methylated by diazomethane and the alcohols chromatographed in the free form or after acetylation. In the case of glycerides, the sodium methoxide catalyzed interesterification products are first neutralized, then taken to dryness and the free glycerol acetylated in the presence of the fatty acid esters and the inorganic salt. The fatty acid esters and the triacetin can be recovered subsequently in the usual way by diethyl ether or petroleum extraction. The fatty acid methyl esters and the triacetin can be separated quantitatively by GLC on a variety of columns.

A simultaneous measurement of the fatty acid and the alcohol parts of the fatty esters introduces a means of identification in the TLC measurement. It also allows comparison of the molar recoveries of the alcohol and the fatty acid and could possibly reveal the unsuspected presence of other alcohols in the natural ester mixture.

3. Use of internal standards

In the not too distant past, the use of internal standard was introduced as a means of last resort, when no specific or general analytical method could be applied. The elegance of the method, however, appears to have escaped general notice. The principle of the measurement is extremely simple and universally applicable[84]. An accurately measured amount of a standard substance of suitable concentration and retention time is added to the mixture of the unknown fatty acid methyl esters or other lipids recovered from the TLC plate and to be examined by GLC. After complete mixing and further concentration of the solution, if necessary, part of the solution is injected into the gas chromatograph and the area response recorded. There is no need to control the amount of the solution actually injected as long as peaks of reliably measurable size are obtained. For purposes of calculation it is assumed that all the sample together with the standard has been injected and that the area response recorded for the standard represents its weight. The weights of the other peaks can then be directly calculated from the weight of the standard. The comparative responses of the standard and the unknown lipid components to be measured should be determined in advance by comparing the areas obtained for weighed amounts of the internal standard, and pure fatty acid esters of known composition. Comparable response between the standard and unknown fatty acid esters can be reliably obtained in most instruments if a foreign fatty acid ester is selected as the internal standard, rather than a hydrocarbon or an alcohol, the recovery or flame ionization response of which may differ significantly from that of the lipid esters to be measured. It has been shown[47,66,67] that such fatty acid esters as the methyl pentadecanoate or heptadecanoate are well suited for this purpose, as their concentrations in common lipid ester mixtures are very limited. The use of these esters also avoids overlapping the standard, with any major fatty acid esters, on any given liquid phase as they move to their appropriate place in the GLC elution pattern. When a potential overlap of the odd carbon number fatty acid esters in the internal standard is to be avoided, the 2-methyl derivatives of fatty acid esters may be used as internal standards. These branched-chain esters emerge just before the corresponding straight chain homologues and give comparable responses in the flame ionization detector[64]. For the analysis of long chain

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triglycerides, a medium chain length triglyceride may be conveniently employed and *vice versa*[6]. It is wise to select, however, a triglyceride which does not contain any of the fatty acids that are present in the glyceride mixture to be analyzed, because then the glyceride mixture, together with the added internal standard, can be transesterified and the fatty acid proportions determined, giving a further check on the recovery of the triglycerides and the accuracy of the measurement. Thus for plasma lipids (steryl esters and triglycerides) tridecanoin has been effective[85] as little or no decanoic acid is found in normal plasma.

Since the accuracy of the final estimate depends upon the knowledge of the exact amount of the added standard, great care must be exercised in the preparation of the standard solution and in its addition to the unknown mixture. Usually a relatively large quantity (100 mg) of the internal standard is weighed out and diluted to 1 l with chloroform in a volumetric flask. This solution may be divided up into 9 × 100 ml volumetric flasks containing 10 mg of standard per flask. For use in GLC, the contents of each such flask can be further diluted to 1 l and again subdivided among 9 × 100 ml volumetric flasks to give chloroform solutions containing 1 mg of standard per flask, which corresponds to 10 μg standard per milliliter of solution. Depending on the final volume of the unknown lipid solution and the amount to be injected into the gas chromatograph, 1 to 5 ml of this standard may have to be added to keep all peaks on scale and of sufficient deflection. For reliable results the peak of the standard should give approximately 50 % full scale deflection and should constitute at least 10 % of the total area. For analyses in less sensitive detectors, more concentrated standard solutions may have to be used.

As the response of the fatty acid esters varies with the chain length of the acids, it is necessary to determine the response ratios of the different molecular species of the lipids, by preparing standard solutions of representative compounds. For this purpose a standard mixture of methyl esters of the acids to be determined is prepared on an equal weight basis and chromatographed. The response ratios of the peak areas of the standard compounds with respect to the internal standard are then determined. For the calculations of the ratios, a base line is constructed under each peak in the GLC record, and the peak area is expressed in square mm for each peak and recorded. The peak area of the internal standard is divided by the peak area of each component. These numbers are the responses or multiplying factors in terms of the internal standard and are recorded in a table. In the analysis of the unknown mixture, a known amount of the internal standard is added to the sample before injection. The peak areas of the sample components are then corrected by the multiplying factor and divided by the peak area of the standard. This may be expressed in the form of an equation as follows (modified from WYNNE *et al.*[78], who used peak heights):

$$\frac{\text{Peak area of component} \times \text{Multiplying factor} \times \text{mg\% of internal standard}}{\text{Peak area of internal standard}} = \text{mg\% component.}$$

If a dilution of the sample has been used a suitable correction factor must be introduced. Various other aspects of analysis by GLC with internal standards have been discussed in considerable detail and appropriate formulae supplied in a review dealing with the gas chromatographic quantification of steroids and their derivatives[86].

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4. Reconstruction of the quantitative composition of the original lipid mixture

In the past, the estimations of the total amounts of individual lipid classes have been made on the basis of most of the components of the molecule except the fatty acids which make up the bulk of lipid molecules. This has prevailed because all lipid classes apparently contain much the same fatty acids and therefore do not provide means for simultaneous quantification and differentiation of individual ester classes. With the availability of chromatographic systems for complete separation of all classes of lipids and exact methods of fatty acid determination, it has become feasible to obtain reliable measurement of lipid classes from the sum of the amounts of the constituent fatty acids[47,54,66,87]. An analysis for the bulk component of a molecule is both theoretically sound and experimentally feasible. Such an assay is much more sensitive than other techniques based on the determination of glycerol, organic phosphorus, inositol, serine, ethanolamine, choline, or cholesterol parts of the molecule[1] and more specific than the estimation of total acyl groups[88].

The calculation of the total quantity of a given lipid class from its fatty acid composition consists of obtaining a quantitative estimate of each of the different fatty acids present. This data is derived from the ratios of the areas of the fatty acid peaks to the area of the internal standard in the gas chromatogram as just described or in any other true or arbitrary units. From the determined amounts of the fatty acids and the knowledge of their molecular weights it is possible to calculate the amount and the molecular weight of a hypothetical average fatty acid. The latter information permits the estimation of the molar concentration of this average fatty acid. Assigning one, two, three or four residues of the fatty acid of the average molecular weight, as the case may be for a given lipid class, to the original lipid molecule, the molecular weight of the total molecule containing the average fatty acid can easily be calculated and consequently the number of moles of the original lipid class estimated. These calculations may be summarized in the form of a formula[47,54], which gives the weights of the original lipid classes in the units of the internal standard:

$$X = \frac{\frac{W_{\mathrm{FAMe}}}{MW_{\mathrm{FAMe}}} \times MW_X}{N_{\mathrm{FA}}}$$

where X is the lipid, the quantity of which is to be determined; W_{FAMe} is the total weight of the fatty acid methyl esters present in the lipid as estimated by means of the internal standard; and MW_{FAMe} is the molecular weight of the average fatty acid methyl ester as determined from the mole percentages and the molecular weights of the individual fatty acid methyl esters. MW_X is the molecular weight of the lipid molecule containing the average molecular weight fatty acid characteristic of each sample. N_{FA} is the number of residues of the average fatty acid per total molecule. The exact molecular weight (MW_X) of the lipid molecule containing the fatty acid of the average molecular weight is calculated by writing down the molecular weight of the lipid molecule containing the fatty acid of the nearest whole number carbon chain and adding or subtracting the weight of the outstanding fraction of a CH_2 unit. The molecular weight of the average fatty acid and the average original lipid molecule could also have been calculated from the knowledge of the chain lengths of the fatty acids and their weight proportions in the mixture.

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It is seen that the detection and correct identification of the chain length of all fatty acids is essential for this calculation, but that the number and location of the double bonds is not so important. The TLC separations must be complete or alternative means available for ascertaining the nature and extent of cross-contamination. Since there is no significant difference in the molecular weight between phosphatidyl serine and phosphatidyl ethanolamine, an accurate estimate of the combined amount of the cephalins may be obtained without actually effecting a complete resolution.

The final result of the analysis is as accurate as the fatty acid determinations, which can be made highly specific and accurate by GLC methods. The mathematical manipulations, although simple and individually readily accomplished, add up to the bulk of the analytical effort and may render multiple analyses impractical. They lend readily, however, to computer programming which simplifies them to the extent of transferring the original data from the print out integrator of the gas chromatograph to the input cards of the computer.

5. Correction of cumulative error

In a closed system in which measurements are taken and then adjusted to fit the restriction $X_1 + X_2 + \ldots + X_n = C$ where the X's are the observed values and C is a constant, the adjusted values and their errors are interdependent. When normalization techniques are used the accuracy and precision of a given observation are likely to be different from the accuracy and precision obtained when the observations are made independently. The effect of normalizing a set of analytical determinations in a mixture of components depends on the amount of each component present, and the variability of the method of measuring each component. In material balance studies such as these, the measurement of certain components of the sum may be much more precise than the measurements of other components. Thus the fatty acid data may be much more precise and reliable than the data on the cholesterol content, or the estimates of the neutral lipids more precise than those for the phospholipids. Also, the estimates for phospholipids may be more accurate when made on the fatty acid methyl esters prepared in the presence of the silica gel rather than following a prior elution where there may have been more chance for oxidative losses.

It has been shown[89] that the standard method of normalizing (dividing each reported percentage by the sum of all reported percentages) can be misleading. It has therefore been recommended that normalizing be done in such a manner that the amount of change in the percentage of a given component due to normalizing is proportional to the standard deviation of the measurement. Only where the standard deviations are proportional to the amount of lipid present will the results of such a normalization be the same as those obtained by merely dividing each determined proportion by the total. Where the standard deviation is independent of the amount present, the effect of normalizing will be much greater. However, where one or more estimates are considerably more precise than the others, acceptable results may be obtained by taking this estimate or estimates as absolutely precise. When the ratio of standard deviations is in the order of 10 to 1, this method gives results practically indistinguishable from those obtained by normalizing in proportion to the standard deviation[89]. On the basis of previous knowledge of the reproducibility of the GLC measurements of various lipid components, it is permissible to take certain results as absolutely correct and calculate the other values by difference. Because of the numer-

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ous opportunities for cross-checks, and for repeated corrections of errors, the long series of measurements necessary for complete analysis of plasma lipids, for example, are essentially free of cumulative error[47].

Since the fatty acid determinations are the most accurate means of estimating the content of the individual lipid classes recovered from the TLC plates, the emphasis is placed upon using these values as the true total amounts. The data obtained from the direct GLC analysis of the steryl esters, triglycerides or the diglyceride acetates derived from phospholipids, are used only for the estimation of the proportions of the individual molecular species within each lipid class. At no stage are these less precise values averaged with the more exact fatty acid data in the product normalizations. The data obtained from the high temperature examination of the high molecular weight components, in the presence of suitable internal standards, in GLC, are used, however, for cross-checking the subtotals and eventually the grand total. When the agreement is good, all the estimates are apparently correctly made and the recoveries appear nearly complete. If the agreement is poor, the fatty acid data is rechecked and taken as absolutely correct. Due to the presence of the internal standard and because of repeated determination of correction factors, there is usually no need to place more emphasis on one type of data than on the other. Methods are available for independent determination of the recovery of most components and any error observed can be immediately corrected, yielding final values of essentially equal accuracy and precision.

4. APPLICATIONS TO NATURAL LIPID MIXTURES

Although only a limited number of applications of the combined system have been made to the complete analysis of natural lipid mixtures, the technique would appear to be universally applicable. Because of familiarity with the operation of adsorption columns and relative resistance towards the innovations introduced by TLC, many projects have been assigned to separations by silicic acid columns, when in fact they could have been completed much faster and at a lower cost if done by TLC. With the recognition of the possible detrimental effects of silicic acid column chromatography upon the stability of cardiolipin[23], phosphatidyl ethanolamine[24] and the plasmalogens[25], more laboratories are expected to rely on TLC for the separation of the lipid classes and will need to consider the systems and applications reviewed here. The applications of combined TLC–GLC methods to the analysis of steroids[86] and to the study of triglyceride structure[12] have been reviewed elsewhere.

(*a*) *Tissue lipids*

DOBIASOVA[59] was one of the first to report on the combined use of TLC and GLC for rapid separation and determination of minute amounts of tissue lipids in a large number of samples. Lipids were extracted from various tissues of rats by ethanol–diethyl ether (3:1) and following TLC separation on silica gel G, the various components were recovered by chloroform–methanol (2:1) elution. It was estimated that at least 85% of the lipids were recovered from the 0.5 mm thick plates. The fatty acid methyl esters were prepared in sealed ampoules by heating with methanolic hydrochloric acid. Total phospholipids, free fatty acids, triglycerides and cholesteryl esters were determined by this procedure. The advantages of this combination for the study of tissue

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lipids were also recognized by MANGOLD AND KAMMERECK[90] who showed that satisfactory results can be obtained by GLC analysis of the methyl esters prepared by transesterification of lipid fractions that had previously been isolated from thin-layer plates. Characteristic fatty acid patterns were observed[91] by this means for each lipid class in many tissues of healthy adult individuals and differences were revealed on comparison with corresponding tissues from healthy infants. FELDMAN AND ROUSER[9] and ROUSER *et al.*[92] isolated lecithin and sphingomyelin from normal and pathological human brains, by DEAE cellulose columns, as a single fraction and used TLC and GLC for subsequent resolution of the two phospholipid classes and a quantitative determination of the fatty acids. The great sensitivity of the GLC determinations made possible the analysis of a few micrograms of a lipid class obtained from a single spot on a two-dimensional chromatoplate. These workers proved that the transesterifications could be safely carried out in the presence of the silica gel from the plate and that there was no need for a prior elution of the separated components. The method was claimed to be of wide applicability.

ROSENBERG AND STERN[68] isolated brain sphingomyelin, cerebrosides and gangliosides by TLC and subjected the recovered fatty acids to GLC. The fatty acids were released from the phospholipids by methanolysis with BF_3-methanol[16]. The presence of silica gel during the transmethylation caused no measurable change in the subsequent GLC patterns of the fatty acid esters. The compounds were located on the TLC plates by brief exposure to iodine. The cerebrosides were resolved on the TLC plates according to their content of hydroxy fatty acids. For the measurement of the ganglioside fatty acid esters by GLC, methyl linoleate was used as internal standard. No destruction of this unsaturated ester was observed under the working conditions when the methanolysis was done under nitrogen and there was no evaporation of reagent due to leakage of the screw cap vials used in the reaction. The values obtained with standards were within 2 % of the known compositions.

A combination of TLC and GLC techniques incorporating the *in situ* transmethylation was used by PRIORESCHI[53,54] for the determination of the individual phospholipids of rat hearts, from normal animals and from animals with experimentally induced cardiopathies. The preliminary separations of neutral and phospholipids originally performed on silicic acid columns were subsequently found to be more efficiently done by TLC[54]. It was observed that the onset of the cardiac necroses was preceded by changes in the concentrations of lecithin and cardiolipin as well as by distortions in the fatty acid compositions of these phospholipid classes.

A rapid micromethod for the analysis of tissue phospholipids and their component fatty acids by means of a combined TLC–GLC analysis has also been described by PARKER AND PETERSON[44], who studied the lipids extracted from 30 g of liver pooled from two adult rats. Complete resolution of all phospholipid classes was obtained by means of a saturation chamber in combination with the solvent systems of SKIPSKI *et al.*[52] and a specially washed silica gel H. Although the recovery of the fatty acids of the phospholipids on GLC was not precisely determined, identical amounts of phospholipid standards methanolized directly and after TLC gave essentially identical GLC peak areas. GLC of the fluorescein sprayed blank areas of adsorbent, of size comparable to those of the reference phospholipids, revealed a minute peak with the same retention time as methyl palmitate.

After examining a variety of saturated and unsaturated lecithins by combination

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acids and the isopropylidene glyceryl ethers by GLC. Rapid acetonation of the glyceryl ethers was obtained at room temperature by adding a drop of perchloric acid to the acetone solution of the ether. The reaction was terminated after 30 minutes by the addition of water. Both the methyl esters and the isopropylidene glyceryl ethers were hydrogenated prior to GLC. This procedure, aside from providing a check on the chain lengths of the unsaturated derivatives, was particularly valuable for detecting branched chain compounds that overlapped unsaturated straight chain derivatives.

The combined TLC-GLC systems have proven equally well suited for the examination of plant lipids. NICHOLS[96] used preparative TLC to obtain individual classes of the polar lipids of *Chlorella*. GLC was used for the determination of the fatty acid composition of the monogalactosyl-, digalactosyl- and sulphoguinosyl-diglycerides and the phosphatidyl choline, ethanolamine and inositol as well as cardiolipin and total neutral lipids. Prior to TLC, solutions of *Chlorella* lipids in chloroform were applied to DEAE-cellulose for a partial fractionation with chloroform–methanol mixtures of increasing polarity. HAVERKATE AND VAN DEENEN[97] used TLC on silver nitrate plates for the subfractionation of phosphatidyl glycerol from spinach leaves and estimated the fatty acid composition of the two subfractions obtained, by GLC. The total concentration of each subfraction was obtained from analyses of lipid phosphorus.

(*b*) *Lipids of blood, lymph and milk*

As early as 1962, GORDIS[42] used thin-layer chromatography for the isolation of the triglyceride fraction of plasma fat particles and determined the fatty acid composition of this lipid class following transmethylation in the presence of the silica gel. The methyl esters were obtained by refluxing the scrapings from the appropriate areas of the TLC plate with dry hydrochloric acid–methanol for 2 h at 120°. The advantages of combining TLC and GLC for the determination of the fatty acid composition of serum lipids were demonstrated by BOWYER *et al.*[22] who analyzed the sera of three pigs. In a comparison between TLC and column chromatographic methods, good agreement was obtained for the values of the fatty acid composition of cholesterol esters, triglycerides and free fatty acids. With phospholipids, agreement for values of lecithin and lysolecithin was moderately good but with sphingomyelin agreement was poor. The chief error was in the gas chromatographic method which gave a consistent error of 5 % for any fatty acid. On the basis of the work of FELDMAN AND ROUSER[9] it would appear that part of the difficulty with the sphingomyelin determination may have been due to insufficiently high reaction temperatures for transmethylation in the presence of the silica gel. Using methyl heptadecanoate as an internal standard and the techniques of TLC described by BOWYER *et al.*[22] and SKIPSKI *et al.*[52], KUKSIS *et al.*[47] determined the distribution of the lipid classes and the composition of the fatty acids in the plasma of 100 university students in the fasting state. The estimates for the total lipids and the individual neutral and phospholipid classes derived by this means were somewhat lower than the average values reported by others and occupied the lower end of the normal range[98]. The estimated proportions of the individual lipid classes, however, agreed with those determined by other methods[99]. The great variations observed in the plasma lipid composition of different subjects were surprising and pointed to the dangers of deriving normal ranges from a limited number of observations on a few subjects. The combined TLC–GLC system has since been

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of TLC and GLC, ZEE *et al.*[87] concluded that the smallest amount of liver lecithin that could be reliably measured by this integrated system was about 0.3 micromoles or 200 micrograms of this highly unsaturated lecithin. This limit is considerably below the 5000 microgram level suggested by BOWYER *et al.*[22] but does not quite reach the nanogram amounts of phospholipids examined by FELDMAN AND ROUSER[9]. ZEE *et al.*[87], however, relied upon elution of the original lipid, saponification and diazomethylation for their fatty acid methylation, and probably incurred losses during the extra manipulations. FELDMAN AND ROUSER transmethylated their esters directly in the presence of the gel.

HOLMAN AND HOFSTETTER[93] used preparative TLC in combination with GLC to determine the fatty acid composition of various lipid classes from whole bovine and porcine reproductive tissues. The separated zones were scraped from the plates and transmethylated with methanolic hydrochloric acid. The unsaturated fatty acids were identified by comparison with the GLC behaviour of a large number of standards of known structure[94]. The relative proportions of the individual lipid classes were estimated by quantitative gravimetric TLC. No attempt, however, was made to separate the individual phospholipid classes.

Combined TLC–GLC analysis has been used by RENKONEN[11] to study the composition of mixed natural lecithins. The diglyceride acetates obtained from eggs, ox brain, and human serum by acetolysis were subjected to preparative TLC on silver nitrate impregnated plates and the recovered subfractions were analyzed by GLC for their fatty acid composition. When the collected data were combined with the results of specific enzymic hydrolyses of the acids occupying the alpha and beta positions of the molecules, a more complete description of the natural lecithin families was possible. The acetolysis was also satisfactorily completed with selected samples of phosphatidyl serine, ethanolamine and inositol as well as cardiolipin, the diglyceride acetates of each of which could be subsequently subjected to the combined TLC–GLC examination. This method has since been extended to the determination of the molecular weight distribution of these phospholipids and their monoene, diene, triene and saturate subgroups by GLC[13]. The combined TLC–GLC system has also been applied[11] to the examination of the acetolysed sphingomyelins, resulting in partial resolution of the different sphingosine bases and their fatty acid esters.

The method developed by BLANK *et al.*[69] for the determination of the lecithin structure also uses combined TLC–GLC analysis, but the lecithins are fractionated as the mercuric acetate adducts. The concentrations of the individual subgroups of lecithins were estimated by GLC of the fatty acids in the presence of an internal standard. Preparative TLC using silver nitrate impregnated plates was profitably combined with GLC by ARVIDSON[15] for the direct analysis of the lecithins from egg yolk and the livers of various species of animals. Each lecithin gave only one spot on rechromatography. The most satisfactory results were reported to be obtained in unequilibrated chromatographic tanks using chloroform–methanol–water (65:25:4, v/v/v) as the developing solvent. The obtained data were in close agreement with the assumption that the majority of the lecithin molecules contain one unsaturated and one saturated fatty acid per molecule. The separations did not permit any conclusions concerning the position of the unsaturated fatty acid on the glycerol molecule.

MALINS *et al.*[95] used TLC to isolate the triglycerides and diacyl glyceryl ethers of the flesh and liver of the dog fish (*Squalus acanthias*) for the determination of the fatty

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extended to the determination of plasma lipid patterns in other subjects following the consumption of controlled experimental diets and has allowed the demonstration of characteristic changes[100].

An integrated system using chromatography on silica gel impregnated glass paper, an internal standard and GLC has been described by BOWERS *et al.*[66] for the determination of the total triglyceride and the triglyceride fatty acids in serum. Calculations of the concentration in serum of each of the individual TG fatty acids is based on the internal standard, heptadecanoate. The concentration of total serum TG was obtained by summation from values of the individually determined fatty acids. The values obtained by this system for total TG compared favorably with values obtained by application of the hydroxamate colorimetric method in combination with silicic acid column chromatography.

Pooled phospholipids from human plasma chylomicrons have been fractionated by TLC by WOOD *et al.*[101] who used GLC only for the determination of the fatty acid proportions. The concentrations of the individual lipid classes were obtained by determination of organic phosphorus. Combined TLC and GLC analyses have been extensively applied by HUANG AND KUKSIS[83] to the determination of the lipid composition of the chylomicron membranes, fat cores and lymph sera of dogs following corn oil and butterfat feeding. The total amounts and the composition of the individual neutral lipid and phospholipid classes were estimated by means of fatty acid analyses using methyl heptadecanoate as an internal standard. The individual lipid classes were recovered from the TLC plates as the methyl esters after transesterification with methanolic sulphuric acid in the presence of the silica gel. The membranes and the triglyceride cores of the chylomicrons were separately isolated by centrifugation and their lipid compositions compared to those of the lymph sera. An identical system of analysis was used[102] for the determination of the lipid classes present in the milk fat globule membrane, the fat core and the milk sera of summer and winter milks.

Preparative TLC was used by MORRISON *et al.*[17] for the separation of mg amounts of the phospholipids derived from glycolipids of milk for analysis of the fatty acid distribution among the various lipid classes. The combined TLC–GLC system was also used for the examination of the phospholipase A hydrolysis products of the diacylphospholipids. Comparisons with the results obtained for phosphatidyl choline from other bovine tissues and from egg lecithin, showed that fatty acids were located much less specifically in milk phospholipids than in the phosphatidyl choline from other sources.

(c) *Other lipids*

MANGOLD[103] has presented an extensive survey of the applications of TLC to the analysis of fats, oils, waxes and their hydrolysis products from microorganisms, plants and laboratory animals and has indicated the cases where the recovered products were reanalyzed by GLC. Most of these TLC-GLC combinations, however, have been of a qualitative nature.

The fatty acid composition of the steryl esters, triglycerides and phospholipids of brown and yellow fat of male albino rats was determined by CHALVARDJIAN[104] by means of combined TLC–GLC analyses. The separated lipids were made visible by exposing the TLC plates to iodine vapor for 30 sec. The zones were scraped off with a brush into tubes fitted with condensers and the contents refluxed with methanol-

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possible to work with single cells, it would appear to be necessary to first perform an extensive tissue, cellular and subcellular fractionation resulting in the isolation of the lipoprotein complexes associated with subcellular organelles of uniform composition.

There would then be a further need to effect a separation of the lipoproteins before the lipid composition could be effectively analyzed. Since the lipoprotein pattern and the lipid composition of a cell may change during maturation or aging, it may be informative to segregate cell populations according to their age. Although no attempts have been made to apply such a scheme to any lipid investigations reported to date, the need for the isolation and analysis of the individual lipoproteins of subcellular organelles has been recognized, and preliminary observations reported[31]. There would appear to be no basis for the suggestion that complete separations of tissue lipids by chromatographic procedures must be considered dubious[30], provided the fate of the original or native lipid complexes has been closely controlled throughout the process of isolation and preliminary separation. The final lipid fractions fulfilling the homogeneity requirements of chromatography can then be related back to the orignal structures whatever their stability under a given set of experimental conditions.

6. CONCLUSIONS

This review has demonstrated the merits of the combined use of TLC and GLC techniques for rapid and essentially complete lipid analyses, and has surveyed the pertinent applications. It is obvious that not all parts of the integrated system have been adequately tested with all kinds of lipids and that there is a danger of uncritical application of the method in unsuitable circumstances. There remains scope for further improvement in the methods of separation and recovery of many components and the investigator must be constantly on guard for artifacts introduced by new batches of separating media and other changes in working conditions. He should not allow himself to be carried away by the apparent theoretical soundness of the general approach.

It should be emphasized that such detailed analyses, no matter how accurate, are unlikely to have much biological or even chemical significance unless the source of the lipids is specifically defined. Confusion and wasted effort is liable to result from work with whole tissues, impure subcellular particles and mixed lipoprotein or glycolipid complexes. It is likely that for an effective determination of the lipid composition of individual lipoproteins or membranous structures, new or improved methods of cell and lipoprotein fractionation will be required before the acquisition and accumulation of the great detail of data afforded by the integrated systems can be fully justified. For many purposes, it may be expedient to ignore most of the potentially available information and to concentrate on one or a few of the components present. Should, however, a complete spectrum of the lipid composition be required, the integrated TLC-GLC system in combination with computer programmed data processing could readily provide it.

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benzene–sulphuric acid (43:5:2, v/v/v) for 2 h at 80° to 90° under nitrogen. The reaction mixture was extracted with petroleum and the petroleum extracts were treated with diazomethane to ensure complete methylation.

ALI[105] used combinations of thin-layer and gas chromatography with internal standards to separate and measure the fecal phospholipids of man on different dietary regimens. Lecithin, phosphatidyl ethanolamine and phosphatidyl inositol together with an unknown phosphorus containing lipid were recognized. For purpose of calculation the unknown phospholipid was assigned the structure of cardiolipin. Large differences were noted in the fatty acid compositions of the individual phospholipids both between subjects on the same diet and between samples from the same subject on different diets. In another study[106] combined TLC–GLC analyses were used to appraise the effect of plant sterols on the excretion of different neutral lipid classes and total phospholipids by other subjects on controlled experimental diets.

Combined TLC–GLC methods have been used to locate the position of eicosatrienoic[107] and docosatrienoic[108] acids in rat phospholipids. The products of enzymic hydrolysis of individual phospholipid classes were separated on TLC plates and the spots of fatty acids and lysophospholipids were treated directly with methanol–hydrochloric acid to obtain the fatty acid methyl esters for GLC.

5. SIGNIFICANCE OF DETAILED LIPID ANALYSES

Much of the recent interest in lipids has been due to the great emphasis placed upon the role of fats in the origin and possible prevention of arteriosclerosis and related lipid diseases as well as to the general availability of superior analytical methods such as gas and adsorption chromatography. Although the enthusiastic search for cures has led to some non-critical use of the incompletely tested methods, a great deal of the adventurism has paid off in new concepts and better understanding of the role of lipid in biological systems. With the more detailed data provided by the advanced instrumentation has come the recognition, that analyses of total lipid mixtures as isolated from crude organ extracts are meaningless in terms of biological structure and function, and that even the methods of preparation of lipid extracts require revision[30]. Lipids, other than those in adipose tissue, occur primarily as components of biological membranes and lipoproteins. Membranes are found at the cell surface (plasma membrane) and in subcellular particles while the serum lipoproteins participate in lipid transport. It is thus essential that individual cellular units be studied in order to define the variations (if any) in lipid composition of different membranes and lipoproteins and relate their lipid composition to membrane and lipoprotein structure and function[26,109]. The possibility that all major enzymatic systems may exist *in vivo* as membrane bound complexes[110] suggests a structural and possibly a functional role for lipid in their activity. The unique ability of lipids to assume well defined and highly specific orientation in aqueous solutions suggests a fundamental role for lipids in the assembly of large molecules during biosynthesis[30,111] and cellular and tissue organization and differentiation[112].

The complexity of individual organs and the possible heterogeneity of the lipids of the various regions of a given organ as well as of the component cells and their subcellular fractions, requires extensive preliminary fractionation of the material before a meaningful analysis of the component lipids can be attempted. Since it is not

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Degumming Vegetable Oils
Lecithin Production

Summary

The separation of some of the nonglyceric components from the vegetable oils can be considered as an intermediate step in the extraction and refining of oil.

Most of these substances can be precipitated and separated by reagents such as alkalis, acids or water. In general water is used when lecithin is produced after the separation. The commercial lecithin is a complex mixture of different phosphatides.

Some of these phosphatides are studied from a chemical point of view, the influence of their presence in the oils and the determination of their contents is considered. A review of different degumming systems and details for the preparation of lecithin are presented.

Degumming of Oils

The crude oils, obtained by the process of pressing and extraction with a solvent of seeds and oil producing fruits, are constituted, in a first approximation, by glycerides (esters of glycerine with fatty acids) and substances that are not glyceric, named generically "nonglyceric components".

These components are of a very varied nature and their presence in the natural oils, is in general, very small. They are in general undesirable though there are some that do not show any prejudicial effect and some others whose presence is beneficial.

The refining of the oil consists of a succession of operations that purify it making it suitable for the intended purposes; this process eliminates most of the nonglyceric components.

These substances could be classified in two big groups: the first one would include those whose presence affects the physical yield of the refining and which can or cannot be eliminated before the refining. In the second group we would include those components for which the elimination can only be achieved, at least with significant results, through partial or total refining of the oil.

Most of the components of the first group coagulate and can be separated by hydration giving as a result a gum-like residue (this originates the name given to the process). This is a previous phase and is independent from the refining process.

The "gums" are constituted by phosphatides, slimy substances not identified, leftovers of organic substances and various components, and of neutral oils, fatty acids and water as a result of the separation process used.

The Phosphatides Group

Work done on fatty substances containing phosphorous in its molecules started with materials isolated from encephalic matters a long time ago (135). A lot of work has been done, especially in the biochemical field, since these compounds seemed to be located in the most vital parts of the live organisms such as the brain, red corpuscles of the blood, liver, etc. There are many articles and monographs on the subject (12), (13), (14), (16), (18), (19), (22), (29), (30), (37), (73), (113), (140).

The study of the composition and structure of these phosphatides is a very difficult task since there is a great variety of them. The difficulty is further increased due to the similarity in their physical properties. Many methods of separation have been tried, some based on the precipitation of these substances with a series of solvents, others adding salts to reduce the solubility (106), (129) or by means of extraction in a counter current or chromatography (126).

We would use the classification given to identify the phosphatides by Hilditch (5). The following groups are distinguished:

I. Compounds that only contain carbon, hydrogen and oxygen:
   a) Esters of higher fatty acids with glycerine (triglycerides).
   b) Esters of 2 higher fatty acids with alcohols other than glycerine (higher aliphatic alcohols, sterols and others).

II. Compounds that contain other elements (phosphorous and nitrogen) besides carbon, hydrogen and oxygen.
   a) Compounds that contain glycerophosphoric acid linked to a nitrogenated base and/or a carbohydrate (phospholipids)
   b) Compounds derived from the (sphingosine) (sphingolipids)
      Derivatives of the phosphoric acid (sphingomyelins) without content of phosphorous (cerebrosides)

While the (sphingomyelins) and the (cerebrosides) are of animal origin and do not appear in plants (2) the phospholipids appear in all of them.

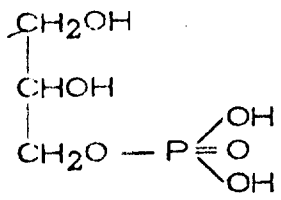
Hildich (5) defines the phosphatides as compounds derived from the α-glycerophosphoric acid in which the remaining hydroxy group of the glycerine are combined with higher fatty acids (as in the glycerides) while the phosphoryl group esterifies with:

a) A nitrogenated base that can be choline (phosphatidyl choline, lecithin), ethanol amine (phosphadidyl ethanol amine) or serine (phosphadidyl serine).

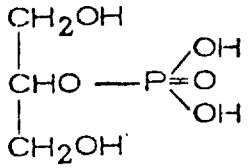
b) The cyclic hexose inositol (phosphoinositides). It can also happen that the remaining phosphoryl group of the phosphoroinositides (derived from the inositol diphosphate) combines with ethanolamine or serine.

The development of the complex molecule of a phospholipid could be simplified as follows:

Starting with glycerine, by introducing the phosphoric acid radical we get the glycerophosphoric acid:

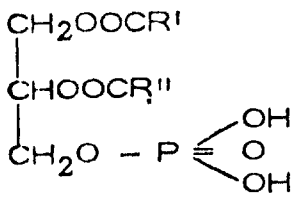


α-glycerophosphoric acid

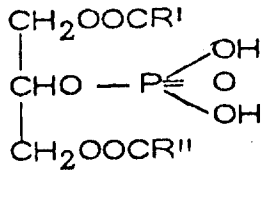


β-glycerophosphoric acid

If the hydroxy groups are substituted by the radicals of the higher fatty acids, which we would represent by the general formula R-COOH we get the phosphatidic acid:



ácido α-fosfatídico

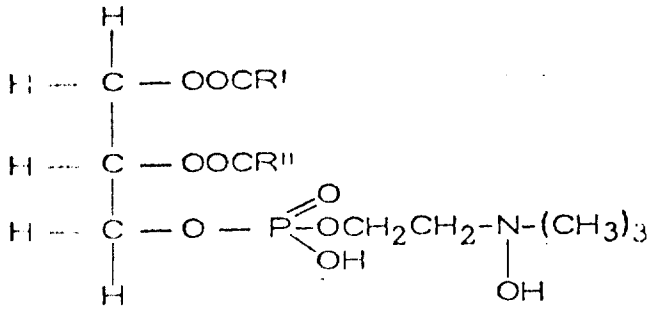


ácido β-fosfatídico

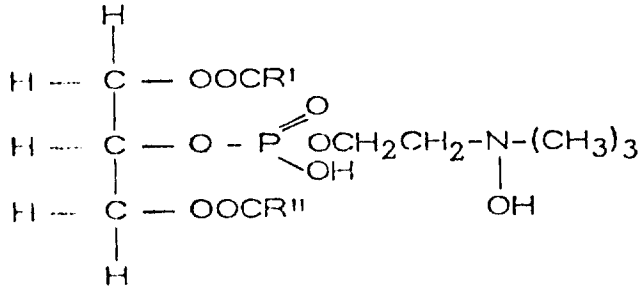
The phosphatidic acids appear naturally in the form of calcium or magnesium salts though they are not very abundant (3).

Lecithins and Phosphatidyl Choline

When the base which esterifies the phosphoryl group is choline ($HOCH_2 CH_2 N(OH_3)_3OH$) we obtained the phosphatidyl cholines, that according to the position of the phosphoric radical originate the α and β forms.

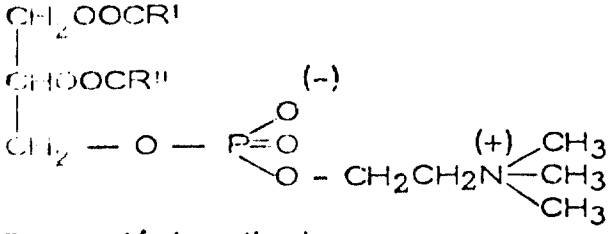


α -lecitina
α -fosfatidilcolina



β -lecitina
β -fosfatidilcolina

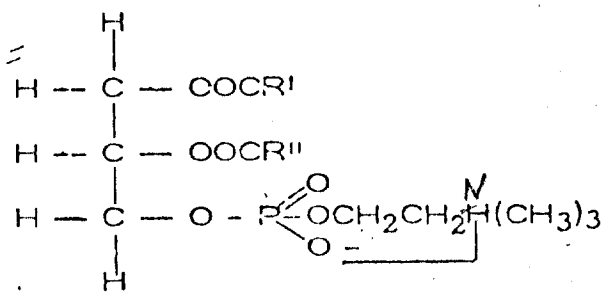
Both formulas correspond to the "hydrated form" which contains at the same time an acid group (the remnant of the phosphoric radical) and a basic group (the quaternary ammonium of choline) (3) though many times they are represented in "the dipolar ionic form" (99), (102):



Forma iónica dipolar

If we observe the molecular distribution of the α-hydrated form, we see that the central carbon is asymmetric (that is, it has its four valences saturated by four different radicals) and this gives place to optical activity. However Dessiuelle (3) points out that it is difficult to measure the rotatory power of these substances since other substances of a very asymmetric nature cling very strongly to the phosphatides during their purification (70), (122), and at the same time the temperature and the solvent used in the purification of the phosphatides modify the rotatory power producing in some cases a partial racemization.

On the other hand the presence, in the same molecule, of two acid groups and a basic one so well defined, seems somehow unstable and one could imagine the formation of an internal salt with the elimination of a water molecule:



α-endolecitina (93)

Nevertheless, it should be noted that according to the percentages of dimyristo-, dipalmitito- and pure diestearophosphatidyl cholines, these seem to correspond to the hydrated form rather than the internal salt or the dipolar ionic form (71).

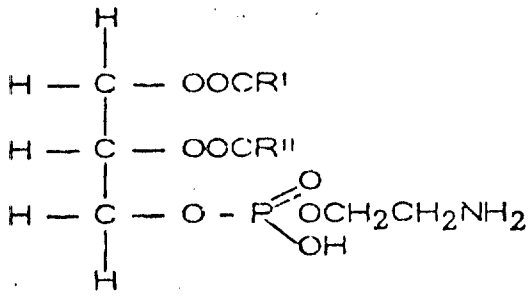
According to the nature of the different radicals R in the previous formulas, we have a family of compounds. If the radicals R correspond to the palmitic or oleic acid (R' and R") the compound could be denominated palmito-oleo phosphatidyl choline or if it was the palmitic acid only (R' = R") it would be dipalmito-phosphatidylcholine.

(Cephalins)

From the group of phosphatides separated from the oil by precipitation with acetone, one fraction (the lecithins) is relatively soluble in ethanol, while

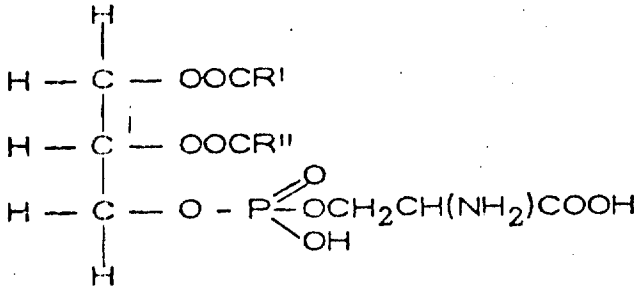
the other is very slightly soluble or almost insoluble. This precipitate is constituted by the so-called "cephalins" that is a mixture, generally quite complex, of different phosphatides.

In the beginning it was assumed that this group was formed by the phosphatides resulting from the esterification of the phosphoric radical of the phosphatidic acid with ethanolamine ($HOCH_2\ CH_2\ NH_2$), that is why sometimes "cephaline" is used to designate the phosphatidyl ethanolamine:



α-phosphatidyl ethanolamine

However, Folch and Schneider show that 40 to 70% of the nitrogen in the cephalines corresponds to a hydroxy amino acid (84), (85), that was the L(+) -serine of formula $HOCH_2\ CH(NH_2)\ COOH$; this acid combines with the hydroxy group of the phosphoric radical forming an ether of the phosphatidic acid.



α-phosphatidylserine

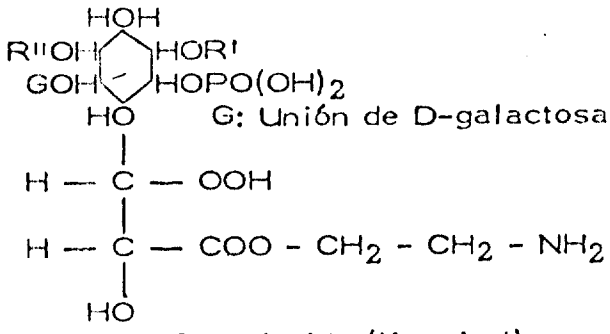
It seems that the phosphatidylethanolamine is a decomposition product of the phosphatidylserine, by decarboxylation (140).

The phosphatidyl choline and the phosphatidylethanolamine contain acid and basic groups that would probably compensate. In the phosphatidyl serine, the acid group predominates and the chemical reactions are those of an acid, and we find them in natural products associated with potassium and sodium ions (3).

Phosphoinositides

We have a third group in the mixture known as cephalins. The first ones to detect the presence of inositolmonophosphoric acid in the cephalin of the soy were Klenk and Sakai (103) and later, by fractioning of this group, Folch and Woolley (86), (87), (89), (138) found the phosphoinositides in the least soluble fraction.

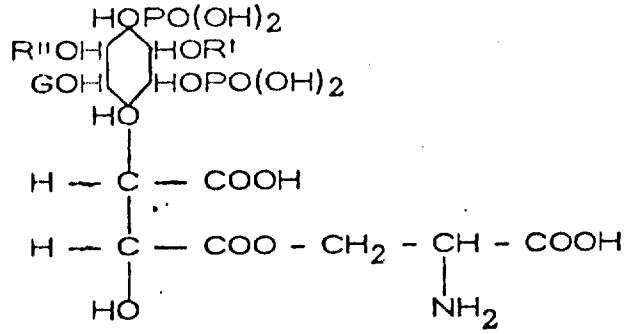
Woolley isolated (139) a compound which he named LIPOSITOL.



- Monofosfonositoide (lipositol)

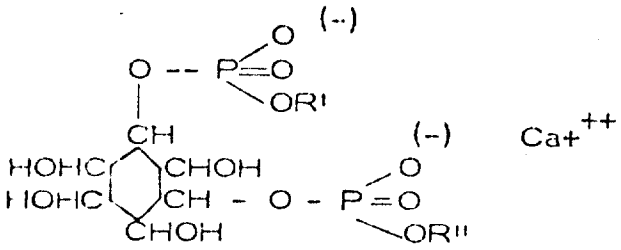
The complete hydrolisis of this compound liberates a mole of the following compounds: mesoinositol, galactose, phosphoric acid, ethanolamine and tartaric acid, with two molecules of fatty acids (3).

The diphosphoinositide was isolated at a later date by Folch (88):



Difosfoinositoide (10)

or

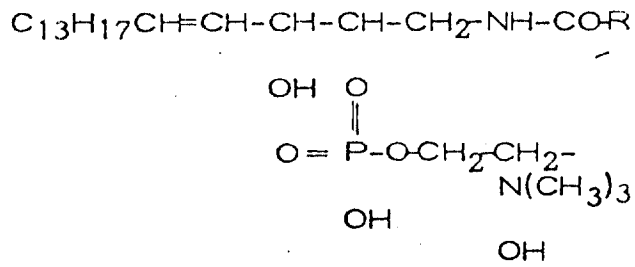


Difosfoinositoide (3)

The structure of these compounds is not known completely but it has been possible to verify that they appear in significant proportions in the phosphatides both of animal and vegetal origin (97), 123), (124), the percentage being 40% in the phosphatides coming from the soybean (127).

(sphingomyelins) and (cerebrosides)

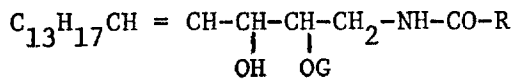
In the phosphophingosides (10) known also as the sphingomyelins (11) the glycerine is replaced by the sphingosine C_{13} H_{17} CH = CH CH (OH) CH (OH) CH_2 NH_2 which saturates one of the OH groups with phosphoric acid, which at the same time esterifies with choline.



sphingomyelins (10)

The sphingomyelins are until now of animal origin only (12). In the case of the galactosphingosides, phosphorus does not appear and they are derived from the sphingosine one of whose hydroxy groups is esterified with D-galactose, while the amine group esterifies with the fatty acids, such as in the sphingomyelins.

That is the way it happens in the cerebrosides:



cerebroside.

Contents of Phosphatides in the Oil Producing Materials

For a long time it was believed (17) that the phosphatides of animal origin were different from those of vegetable origin. Levene and his collaborators were the first ones to isolate pure lecithin from soy (105), (109) working with the phosphatides of this seed (106), (107), (108).

The amount of phosphatides present in the different seeds is in general low (it seldom goes above 0.5%) and its percentage varies greatly, not only with the kind of seed but also with the extraction methods used, that is why we find in the literature very different percentages for the same seed.

As indicated by Stanley (10), the proportion of phosphatides in the seeds varies in direct relationship to the content of proteins instead of that of the oil, soy being a typical example (high in proteins, high in phosphatides) and copra (high content in oil, low content in phosphatides.

Phosphatide content of some oils according to Kaufmann (45).

Oil	% Phosphatides
Cotton	0,7 - 0,9
Rice	0,5
Oats	1
Peanut	0,3 - 0,4
Barley	3,4 - 4,2
Rye	1,3 - 4
Rape (colza)	0,1
Linseed	0,3
Corn	1 - 2
Millet	0,2
Sesame	0,1
Soy	1,1 - 3,2
Wheat	0,08 - 2,0

Lishkevich (111), (112), found that cotton was the seed containing the highest percentage of phosphatides, superior even to soy though the characteristics of the phosphatides for both seeds are very similar (54). The ones from cotton are more stable probably due to the lower iodine index since according to

Thurman (65), (66), the iodine index in commercial phosphatides of cotton is 60 to 70, while in soy,it reaches 90.

Cephalins are the principal constituents in the phosphatides of rape (colza), peanut (with 64.3%) of cephalins and 35.7% of lecithins) and sunflower (56), (with 61.5% of cephalins and 38.5% of lecithins). On the other hand, the sesame oil (56), has the highest percentage of lecithins of all the oils coming from seeds (52.2% of lecithins and 40.6% of cephalins, in the case of linseed is 36.2%.

Degumming Process

From what we have discussed before we could define degumming as a series of operations prior to refining (or following extraction and obtaining of oil) and in which the gums or phosphatides separate before and independently of the form of elimination of the fatty acids, regardless of whether the gums are going to be used or not in the preparation of the commercial lecithin.

Consequently, the process basically consists in causing separation of the gums inside the oil mass, by the action of agents such as water, vapor, acids, etc., agglutinating those precipitates and separating them based on differences in specific gravity.

The product obtained consists of a series of phosphatides, some of them in very small proportions, and other substances of a complex and little-known nature, and of some solid residues from the extracted seeds according to the process employed.

Due to the gum-looking aspect of these masses the generic name of gum is given without going any deeper into the elucidation of their composition.

According to the quality of the gums obtained and the process used in their separation it is possible to prepare, starting from them, by purification or dehydration, a commercial product called lecithin that is a mixture of phosphatides (lecithins, cephalins, phosphatides of inositol), oil and fatty acids.

The degumming is not always performed as a separate process or independent of the refining. In general the volume of the gums present in the oil and the possible utilization or commercialization of the product obtained is observed. If the percentage of these materials in the oils is not significantly noticeable, they are directly subject to refining and in some cases even if the

percentage is relatively high they are refined without previous degumming as in the case of the alkaline refining.

In general, in oils with a high gum content, degumming is done since it facilitates the refining process and in the opinion of some investigators and technical people, it reduces losses in the refining by direct use of caustic soda, due to the emulsifying action of the phosphatides in alkaline solution which increases the percentage of oil occluded in the paste.

Also degumming is sometimes enough for some technical oils as in the case of linseed for varnishes (1).

Fash (83) studied the effect of the phosphatides of "various oils on the necessary conditions for the alkaline refining of crude oil.

In spite of the multiple applications of commercial lecithin since its discovery as a sub-product in the oil extraction, it continues to be a nuisance in the extractors, since only 10 to 30% of the total lecithin obtained goes out on the market.

Nevertheless the market may require, as it is in most cases, that oils such as soy, cotton and linseed be degummed to a maximum content value in phosphatides according to the types of commercialization.

The gums separated from the oils (by hydration in most cases), when they are not used for preparation of commercial lecithin, are often added while wet to the extracted flour (in general, mixing it with the flour in the second or third floor of the solvent extractor - toaster, according to designs, or even better in the toaster, if this is an independent apparatus).

This represents an increase in fat content of the flour which improves the food value, and at the same time it helps to reduce the dust eliminated in the handling of the flour.

When the lecithin is not going to be processed after obtaining it, it is convenient to have the degumming centrifuge and the pump as close as possible to the place where they are going to be used to avoid the problems due to long distance traveled by the pipes (or tubes) which causes the lecithin to dry out or solidify, plugging the system. In any case, it is convenient to run vapor in the rubber tubes, and to clean the tubes with hot water when the system has been idle for long periods of time.

The degumming stage, independent of the refining process, can take place in the oil extraction plants and as it was mentioned before, the separation of the phosphatides is done by hydration or precipitation although the elimination is not complete (or total). For some physical reason it has not been possible to eliminate more than 90% of the phosphatides (52) due to the fact that the addition of water to these compounds is not complete.

Degumming by Hydration

This is the most commonly used procedure to separate the gums from the oils. The phosphatides, proteins and other impurities of colloidal nature are soluble in the dired crude oils, as real solutions or in a colloidal state, but when water is added to these oils, these materials hydrate and their weight increases, separating from the oil mass as coagulates easily agglomerated and separable by decantation or centrifugation.

Where the gums agglomerate, they engulf a percentage of oil that varies according to the conditions under which the coagulation and separation took place. It seems that the percentage of water used, and the temperature and efficiency of the centrifugation are the factors directly related to the oil retention.

The molecule of a phosphatide presents two very differentiated zones: one occupied by the fat chains (from the fatty acids), of non-polar character, and the other formed by the phosphoric radical and the nitrogenated bases that is of a polar type. These two different tendencies within the molecule confer to the phosphatides a very peculiar physical-chemical behavior which is more accentuated when the distance between these two groups is larger.

The polar groups are oriented towards the water while the hydrocarbon chains run away from it, creating a parallel molecular orientation. The non-polar region attracts the hydrocarbon chains, apparently due to Van der Waals forces (3) and a second chain of phosphatide molecules is formed; they have the same tendency towards the water, identical layers are formed (77) as a function of the number of water molecules.

When the phosphatides are in the presence of glycerides (78), it is different since now the groups are not only constituted by water and phosphatides but also by the glycerides. The energy of interaction varies according to the proportion of phosphatides and glycerides.

Desnuelle (3), distinguishes two maximums: the first clearly differentiated (at approximately 70% of phosphatides and 30% of glycerides) and the second, less precisely at 30% of phosphatides and 70% of glycerides.

The formation of the more stable mixed layer corresponds, as should be expected, to the maximum energy of interaction. So Desnuelle concludes that "any mixed layer formed spontaneously on the surface of the water must contain approximately 70% of phosphatides and 30% of glycerides. When a diluted solution of phosphatides in glycerides is treated with a small amount of water and then dried,it contains the previous proportions.

The mass of phospholipids precipitated with water is formed, according to Jakubowski (41), by a double monolayer of water molecules surrounded by a monolayer of lipids constituted by phospholipids and a 2.3% of glycerides when oil of fresh soy is hydrated,or 20-30% of glycerides when the oil of hydrated soy was partially hydrated.

In its basic form,the stages of hydration, centrifugation and drying are the ones followed in the manufacturing of commercial lecithin from the crude oils. Consequently the appearance of glycerides in the unfinished lecithin does not indicate, a priori, a deficiency in the process or a loss by emulsification, but that a great number of glycerides had taken an active part in the formation of the so-called "swollen figures". Because of this it is not possible to avoid their presence though its percentage is more or less variable according to the conditions during the first two stages (hydration and centrifugation).

Efficiency of Degumming by Hydration

Not all the gums present in crude oil are precipitated by water though their percentage is very small. We can consider two groups called hydrophilic groups; to this belong the gums that can be hydrated or precipitated by water,and another group, the non-hydrophilics,to which the rest of the gums belong.

Mattikow (48) calls the first ones ("lipoids A") and the second ("lipoids B"), estimating that in the case of the crude oil from soy the percentage of lipoids B is of the order of 5 to 10%, being necessary for its separation, strong caustic solutions on the order of 20° Be.

Usually the amount of water needed in the hydration varies from 0.5 to 3%, this percentage being determined by previous tests in the laboratory.

The amount of oil retained by the gums, independently of the more or less fixed percentage due to the formation of the "swollen figures", seems to be in direct relation to the amount of water used, the arrangement of the phosphatide-water-glyceride layer being also influenced by it.

Beyond certain limits, and for the same type of phosphatides, a higher percentage of water used corresponds to a higher volume of gums separated, but the losses of oil due to retention are also higher. Smith (59) considers that a treatment with 1% of water, or less, would produce gums which would not dissolve, more than 15 to 20% of the oil. On the other hand, Maetikow (48) estimates the oil occluded in the gums is approximately of the order of 30 to 35% if the percentage of added water is 1.5 to 3%, and the separation has been done by centrifugation.

In precipitation of phosphatides, lecithin contains 5 molecules of water for eachphosphorous atom while cephalins use 40; in general the phosphatides of soy, precipitate with 7 to 9 water molecules per atom of phosphorus (41).

The temperature also plays an important role in the separation probably more directly related to the centrifugation in itself (effect over the difference in specific weights between the hydrated gums and the oils, in the same manner over the viscosity) than with the hydration or coagulation.

As a general range, the temperature of separation is between 55°C and 82°C. In the case of the soy oil, it seems that the optimal temperature of centrifugation is 75°C, being possible to observe in the yield a possible redissolution of the precipitated gums above this temperature and a diminution in the yield of the separation at lower temperatures.

Burnasheva and Sterlin (28) observed that heating at 60°C of solutions of phosphatides in oil, diminished the hydrophilic and this diminution was more accentuated, the higher the temperature. At the same time, the elimination of phosphatides by hydration would diminish in the case of solutions of oil that had been treated, since the phosphatides yield is lower, the longer the heating.

Another factor that plays a role in the hield of separated phosphatides is the reaction time or agitation during the hydration. According to Wittcoff, this time of agitation can vary from 15 minutes to an hour (12), generally being 30 minutes. (42)

Preparation of the Commercial Lecithin

The crude oil from soy is almost exclusively the source of precipitated gums for the preparation of the commercial lecithin, generally called "soy lecithin".

The gums obtained from cotton oil due to their resinous character and dark appearance do not have at present a commercial use, being added to the extracted flour when they are separated from the crude oil.

Neither are the gums from the linseed oil useful although degumming is frequently done especially for the preparation of technical oils. With the purpose of obtaining a good yield in the degumming, it is advisable to adjust as closely as possible the percentage of hydration water. Kantor (44) estimates that the specific weight of the linseed oil is a function of its content in gums, so that knowing the first, it is possible to adjust the percentage of water in the treatment. This percentage can vary from 0.2 to 2% for specific weights of 0.9315 to 0.9340, respectively.

A typical soy oil, according to Bayley (11) would yield approximately 3.5% of gum-like material consisting of 25% water and a 75% products soluble in oil of which approximately 1/3 is oil and the rest insoluble in acetone (basically phosphatides). As it has been said before, most of the commercial lecithin comes from the soy oil and specifically that extracted by solvents, since the high temperatures used in the hydraulic pressure or expulsion methods tend to decompose the phosphatides (12).

Myers (53) has a system of degumming and preparation of lecithin. These processes can be considered to correspond to the diagram in Figure 1.

With the purpose of obtaining as clear a lecithin as possible, the crude oil from the extraction of the storage tanks is subjected to a filtration before hydration using around 0.06% of substances to help in the filtration, these being mixed in the feeding tank of the filter that is in general of the horizontal type and vertical plaques with a metallic mesh.

Normally, only one filter of a capacity calculated to handle more than the daily production of oil is used and, the volume of the deposit of collected oil filtered at the same time as the one of the mixture of oil and the one used in helping the filtration, are calculated in order to contain more than the oil in transit that is handled during the cleaning of the filter which is

done every 24 hours, making the process continuous. The oil temperature and the amount of water is adjusted, and once the oil is added and the mixture homogenized, it is subjected to a retention time, with light agitation, for the purpose of facilitating the separation of the gums in the oil. This time of retention is estimated as 20 to 30 minutes for the soy oil, calculating the volume of the hydration tank in relation to the capacity of the installation.

The exit from the hydration tank is usually done with tubes in the shape of a lyre or an inverted U in which the upper level corresponds to the maximum time of retention (or volume inside the tank) that has been calculated as previously mentioned. Some communication valves between the two branches of the tubes allow variation of the time of contact as a function of the level maintained in the hydration tank.

The mixture of gums and oils is sent to the centrifuge, where the separation of the gums and oil takes place; from there the oil is sent to the vacuum dryer to eliminate the water, and finally to the storage tanks.

On the other hand, the gums, after being homogenized, are sent to the lecithin vacuum dryer that, in general, is of the type having a fine film that is laminated by the action of two coaxial cone trunks, with an adjustable layer width and with heating by a steam jacket.

Jakubowski (98) gives a description of a pilot plant for degumming and Langhurst and Stanley (6) describe the process of preparation of commercial lecithin which starts in the dehydration tank in which the gums are heated and agitated at 65-71^{o}C under 70 cw of vacuum until the humidity contents is reduced below 0.5%.

The dry lecithin, at 110-115^{o}C approximately, comes out of the dryer as a fluid allowing its pumping to the receiving depots that are generally provided with electric heat or steam jackets, and they have an agitator that allows the homogenization of the lecithin in case substances are added to modify its physical or chemical properties.

One of the factors that in principle conditions the quality of the lecithin obtained is its content of substances insoluble in benzene (B1); this fraction is basically constituted by the fine parts of the flour that passed through the filter in the stage of previous clarification.

The maximum amount of B1 allowed in commercial lecithin is 0.3%; nevertheless, if the percentage is significantly over 0.1%, the lecithin would appear cloudy and below that limit, it would appear clear and shiny. On the other hand, it seems that if the oil subjected to hydration is fresh and has not been stored for a long time, the quality of the lecithin improves significantly. (11).

The dry lecithin obtained by the method previously described is known as commercial lecithin or crude lecithin and according to Sullivan (62) using air-tight centrifuges at 3-4 K/cm^2, it is possible to obtain values as high as 80% insoluble in acetone (AI).

The abundance in phosphatides would depend principally on the method followed for the precipitation and separation of the gums and the percentage of water used as indicated before. Values of 66% of AI as indicated by Bailey (11) or 65% according to data given by Stopper (61) are quite frequent.

The commercial lecithins are not constituted exclusively, not even principally by this type of phosphatides but, are a mixture of lecithins, cephalins, phosphatides of inositol, etc., and fatty acids and oil which are believed to exercise a stabilizing action in these products sensitive to humidity and oxygen (12).

The series of compounds that, in greater or lesser amounts form part of this mixture could be more extensive if it was not that the extraction of the phosphatides together with the oil, in the continuous processes by solvents is quite incomplete. This deficiency can be attributed to two factors: the type of solvent used (it always acts in a selective way), and the humidity of the seed subjected to extraction - the humidity is kept as close as possible to 11%, and this also exercises a selective action in the extraction.

According to Beckel and collaborators (25), the solvent used in the extraction does not only affect the acidity but also the contents in gums in the oil obtained.

Effect of the solvent of extraction on the acidity values and "breakage" of the soy oil (25).

Solvent	Acidity Index	"Breakage"
Dichloroethylene	0,52	-
Trichloroethylene	1,04	1,00
Carbon tetrachloride	0,44	0,06
Isopropanol	0,95	0,86
Ethanol	0,6	0,00
Ethanol (residue)	0,24	0,02
Hexane	0,50	0,50

The soy oil contained in the gums separated by precipitation of this oil, could or could not be replaced by a more stable oil such as peanut oil, coconut, hydrogenated oils or cocoa butter (12). The process followed in these cases consists in dissolving the oil in acetone, separating the solution formed and adding the new oil. It is also possible not to add any oil and leave the phosphatides in a state more or less free after more than one wash with acetone, but the product is very unstable and must be stored very carefully and be used very soon.

A more economical technique than the use of acetone for the purification and concentration of the phosphatides produced by the degumming of the oils, has been proposed by Garmash (38) and consists of dissolving the "lecithin" in hydrogenated fat at 95-100°C, centrifugating to eliminate the insoluble substances at 80/85°C, followed by precipitation by cooling at 40/45°C.

The physical and chemical properties of the natural lecithin can be modified by manipulations performed during the separation process or after it as we will see in the following section.

Bleaching of the Lecithin

Even under excellent conditions, the natural lecithin appears grayish, more or less dark, the color depending in principle on the variety of the seeds in the process and the quality of the oil obtained.

With the purpose of diminishing this dark color of the commercial natural lecithin and to make all the types uniform, independently of the source of origin of the oils, many bleaching agents have been proposed, among which hydrogen peroxide stands out. Hydrogen peroxide is added to the phosphatides emulsion before drying, according to Bollman's patent (75).

Normally, hydrogen peroxide can be added to the tank for homogenization of the gums, immediately before drying using a solution 30-35% in variable proportions that can reach 2% in relation to the phosphatides. The use of this bleaching agent is also described in patents given to Bollman and Bollman-Schwieger (74, 76), citing that hydrogen peroxide increased the capacity of the phosphatides for emulsification.

According to Thurman (67), when hydrogen peroxide is used as a bleaching agent, the phosphatides obtained have a light color, the bleaching having taken place in the hydration stage.

The use of this bleaching agent leads to the type of lecithin called "single bleached" and in some instances its action is reinforced by the use of a second agent, generally benzoyl peroxide which originates a second type of lecithin called "double bleached".

The use of benzoyl peroxide as a bleaching agent appears in the literature (94, 126), which indicates that its use leads to a finished product of a smoother consistency.

When a double bleaching of the lecithin is performed, approximately 0.1% of commercial hydrogen peroxide (p.e. 1.123) and 0.008% of dry benzoyl peroxide are added to the natural product in relation to the oil in the degumming process.

Other agents have been proposed such as the sodium chlorite, as indicated by Greenfield (92), or fatty peroxides proposed by Epstein (88).

Among the physical means of bleaching that have been proposed are clays and absorbent earths by Markman and Vuishneprolskaya (115), and the activated soil by Marmon and Moyer (116), recommending bleaching in the intermediate phase, in the process of extraction by solvent. Julian and Iveson (100) patented a process in which the mixture of the soy oil is treated with a clay of acid type and then another is added of an alkaline type, although the phosphatides are precipitated before the addition of the second.

Other Modifications of the Natural Lecithin

Besides the color, another physical characteristic that is modified or can be modified is the consistency. This can be of plastic nature, the form in which the natural lecithin is mormally obtained, or fluid. Both types are differentiated by their content in fatty acids and the conversion of the first into the second is done by adding 2 to 5% of a mixture of fatty acids in its plastic form (11).

According to the composition of the lecithin obtained by degumming of the processed oil, the fluid grade is prepared in the same storage tanks, by addition and mixture of the convenient proportions of soy oil or fatty acids (in general also of soy).

In regard to the chemically modified lecithins, the type of lecithin that can be dispersed in water (called "water dispersible") results from the addition and mixture of 8 to 10% of an emulsifying agent and 4 to 5% of hexylene glycol to the doubly bleached fluid lecithin.

The emulsifying properties of the lecithin whose oil content is increased to 30-40% by addition of soy oil, can be increased by the addition of aromatic alcohols: propyl gallate and stearate of polyoxyethylene (51).

Types of Lecithin

Six types of commercial lecithin can be distinguished according to whether they are natural, bleached in each of their two versions, are plastic or fluid.

Types of Soy Lecithins

Characteristics	Plastic	Fluid
Moisture Content	1% max	1% max
Insoluble in benzene	0,3% max	0,3% max
Insoluble in acetone	65% min	62% min
Acidity Index	30 max	32 max
Viscosity, poises at 25°C (b)	-	150 max
Penetration (c)	22 mm max	-

The Gardner color (in a solution of 5% mineral oil), is respectively 10, 7, and 4 in maximum values for the three types: natural, single and double bleached.

a) By distillation intoluene during two hours or less (AOCS, Ja 2-56)

b) By means of a conventional viscometer or by the bubble system and AOCS time method Ka 6-59 assuming density equal to one.

c) Using the Precision cone 13J25, and the penetometer 3510; with the sample kept at 25°C for 24 hours.

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1549

Desgomado de aceites vegetales Fabricación de lecitina

J. Lajara

Resumen

La separación de algunos componentes no glicéridos de los aceites vegetales se puede considerar como un proceso intermedio entre la extracción del aceite y su refinación. La mayor parte de estas substancias se pueden precipitar y separar mediante el concurso de reactivos tales como álcalis, ácidos o agua, empleándose generalmente este último cuando a la separación sigue la preparación de lecitina comercial, que es una mezcla compleja de diversos fosfátidos.

Se estudian algunos de estos fosfátidos desde el punto de vista químico y se considera la influencia derivada de su presencia en los aceites, así como la determinación de su contenido en los mismos, con una revisión de diversos sistemas de desgomado y detalles de la preparación de lecitina en sus diferentes tipos.

Desgomado de aceites

Los aceites crudos, tal como se obtienen por los procesos de prensado o extracción por disolvente a partir de las semillas y frutos oleaginosos, están formados, en una primera aproximación, por glicéridos (ésteres de la glicerina con ácidos grasos) y substancias que no lo son, que reciben la denominación genérica de "componentes no glicéridos".

Estos componentes son de naturaleza muy diversa y su presencia en los aceites naturales es, normalmente, muy reducida. Por lo general no son deseables, aunque hay algunos en los que no se aprecia acción perjudicial alguna e incluso en otros su presencia es beneficiosa.

Mediante la refinación, se sucede una serie de operaciones, tendientes a purificar el aceite hasta hacerlo apto para los fines a que se destina, y que lleva aparejada la de la mayor de estos componentes no glicéridos.

Estas substancias se podrían clasificar en dos grandes grupos: el primero comprendería aquellas cuya presencia afecta al rendimiento físico de la refinación y que pueden o no eliminarse previamente a la misma. En el segundo grupo clasificaríamos a aquellos componentes cuya eliminación, al menos con resultados notables, sólo puede efectuarse mediante la refinación, parcial o total, del aceite.

El primer grupo es en su mayor parte, coagulable y separable por hidratación, liberando una masa de aspecto gomoso, que da nombre a este proceso de tratamiento del aceite, como fase previa e independiente de la refinación.

Estas "gomas" están constituídas por fosfátidos, substancias mucilaginosas no identificadas, restos de materia orgánica y compuestos diversos, así como aceite neutro, ácidos grasos y agua, como resultado del proceso de separación empleado.

El grupo de los fosfátidos

Los trabajos con materias grasas que contienen fósforo en sus moléculas, comenzaron con materiales aislados de masas encefálicas, hace ya mucho tiempo (135). Se ha trabajado mucho, especialmente en el campo bioquímico, dado que estos compuestos parecen radicar en las partes más vitales de los organismos vivos, tales como el

cerebro, corpúsculos rojos de la sangre, hígado, etc., por lo que existe una buena serie de trabajos y monografías sobre el tema, (12), (13), (14), (16), (18), (19), (22), (29), (30), (37), (73), (113), (140).

El estudio de la composición y estructura de los fosfátidos es una tarea ardua dada la gran variedad de los mismos, aumentada la dificultad por el hecho de que, las más de las veces, las propiedades físicas de los distintos compuestos son muy parecidas. Se han ensayado diversos métodos de separación, basados unas veces en la precipitación de estas substancias a partir de una serie de disolventes, mediante adición de sales para rebajar la solubilidad (106), (129) o mediante extracción en contracorriente y cromatografía (125).

Para la situación e identificación de los fosfátidos dentro del gran grupo de substancias en cuya composición entran los ácidos grasos superiores, tomaremos la clasificación dada por Hilditch (5), que distingue los siguientes grupos:

I Compuestos que contienen solamente carbono, hidrógeno y oxígeno:

a) Esteres de ácidos grasos superiores con glicerina (triglicéridos)
b) Esteres de ácidos grasos superiores con alcohol distintos de la glicerina (alcoholes alifáticos superiores, esteroles y otros).

II Compuestos que contienen otros elementos (fósforo y nitrógeno) además de carbono, hidrógeno y oxígeno:

a) Compuestos que contienen ácido glicerofosfórico unido a una base nitrogenada y/o un carbohidrato (fosfolípidos).
b) Compuestos derivados de la esfingosina (esfingolípidos):

Derivados del ácido fosfórido (esfingomielinas) con contenido de fósforo (cerebrósidos).

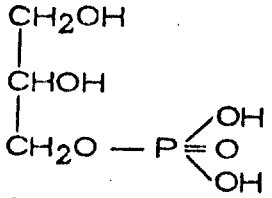
Mientras las esfingomielinas y los cerebrósidos son de origen animal y no aparecen en las plantas (2) los fosfolípidos aparecen en casi todas.

Hilditch (5) define los fosfátidos como compuestos derivados del ácido α-glicerofosfórico en el que los dos grupos oxhidrilo restantes de la glicerina están combinados con ácidos grasos superiores (como en los glicéridos) mientras que el grupo fosforilo se esterifica con:

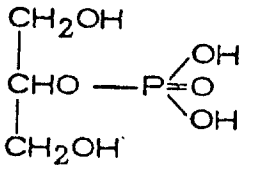
a) Una base nitrogenada que puede ser la colina (fosfatidilcolina, lecitina), etanolamina (fosfatidiletanolamina) o serina (fosfatidilserina).
b) La hexosa cíclica inositol (fosfoinosítidos). También puede ocurrir que el grupo fosforilo restante de los fosfoinosítidos (derivados del difosfato de inositol) se combine con etanol-amina o serina.

El desarrollo de la compleja molécula de un fosfolípido podríamos simplificarlo como sigue:

Partiendo de la glicerina, por introducción del radical del ácido fosfórico, llegamos al ácido glicerofosfórico:

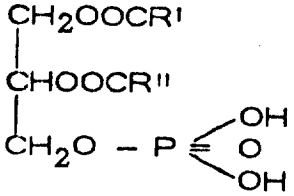


ácido α-glicero-fosfórico

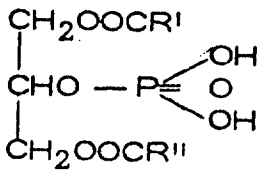


ácido β-glicero fosfórico

Si los grupos oxhidrilo se substituyen por radicales de ácidos grasos superiores, que representaremos por la fórmula general R-COOH, llegamos al ácido fosfatídico:



ácido α-fosfatídico



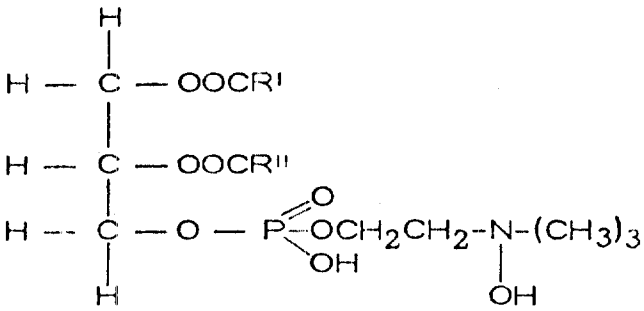
ácido β-fosfatídico

Los ácidos fosfatídicos se encuentran

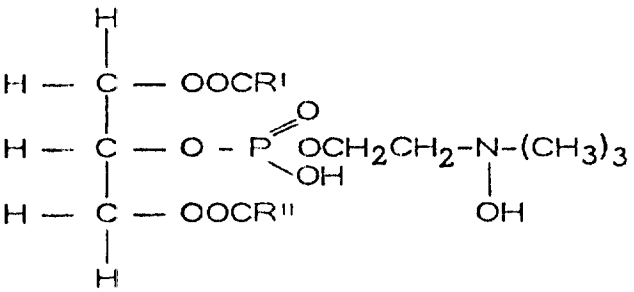
en la naturaleza en forma de sales de calcio y magnesio, aunque no aparecen en forma extendida (3).

Lecitinas o Fosfatidilcolinas

Cuando la base que esterifica el grupo fosforilo es la colina ($HOCH_2CH_2N(CH_3)_3OH$) se obtienen las fosfatidilcolinas, que según la posición del radical fosfórico originarán las formas α o β .

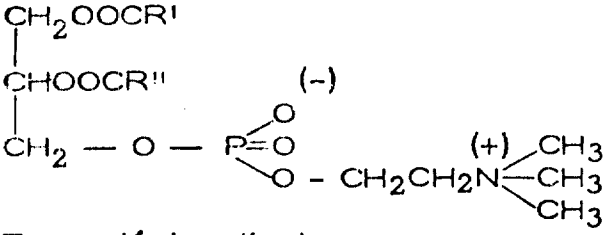


α -lecitina
α -fosfatidilcolina



β-lecitina
β-fosfatidilcolina

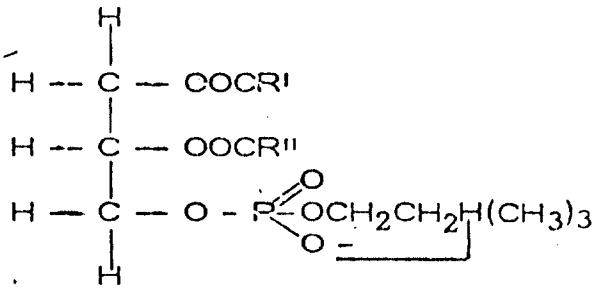
Ambas fórmulas corresponden a la llamada "forma hidratada", que contiene al mismo tiempo un grupo ácido (el remanente del radical fosfórico) y un grupo básico (el amonio cuaternario de la colina) (3), aunque muchas veces se represente en la "forma iónica dipolar" (99), (102):



Forma iónica dipolar

Observando la distribución molecular de la forma hidratada α, vemos que el carbono central es asimétrico (esto es, tiene saturadas sus valencias por cuatro radicales distintos) lo que lógicamente habrá de dar lugar a una actividad óptica. Sin embargo Desnuelle (3) opina que es difícil medir el poder rotativo de estas substancias debido a que otras de tipo muy asimétrico se aferran tenazmente a los fosfátidos durante su purificación (70), (122), a la vez que la temperatura y el disolvente empleado en la purificación de los fosfátidos influyen, de forma variable, sobre el poder rotatorio, llegando incluso a veces, el mismo proceso, a ocasionar una parcial recemización.

Por otro lado la existencia, en la misma molécula, de dos grupos ácidos y básico tan definidos, parece un tanto inestable, y cabe pensar que se originará una sal interna o endosal, con pérdida de una molécula de agua:



α-endolecitina (93)

No obstante hay que hacer notar que, de acuerdo con los porcentajes de composición de dimiristo -, dipalmito - y diestearofosfatidilcolinas puras, éstos parecen estar más de acuerdo con la forma hidratada que con la sal interna o la forma iónica dipolar (71).

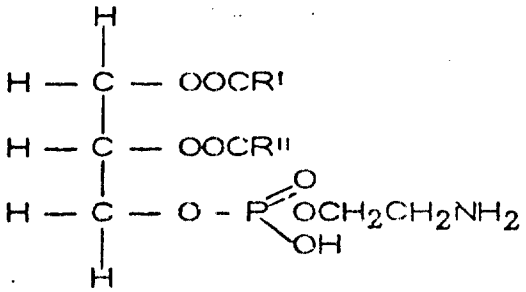
Dados los diferentes radicales R que pueden entrar en las anteriores fórmulas, tenemos una familia de compuestos, de forma que, por ejemplo, en el supuesto de que los radicales R correspondieran a los ácidos palmítico y oléico (R^I y R^{II}), el compuesto se podría denominar palmitooleo-fosfatidilcolina o, si sólo se tratara del ácido palmítico ($R^I = R^{II}$) sería dipalmito-fosfatidilcolina.

Cefalinas

Del conjunto de fosfátidos que se

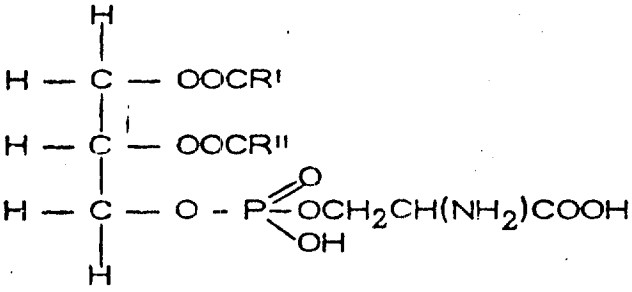
separan del aceite por precipitación con acetona, una fracción (las lecitinas), es relativamente soluble en etanol, mientras que la otra fracción es muy poco soluble o casi insoluble. Este precipitado comprende las llamadas "cefalinas" que son una mezcla, generalmente bastante compleja, de diferentes fosfátidos.

En principio se pensó que este grupo estaba formado por los fosfátidos resultantes de la esterificación del radical fosfórico del ácido fosfatídico con la etanolamina ($HOCH_2CH_2NH_2$), de ahí que a veces todavía se utilice "cefalina" para designar la fosfatidiletanolamina:



α-fosfatidiletanolamina

Sin embargo Folch y Schneider mostraron que del 40 al 70 % del nitrógeno de las cefalinas correspondía a un hidroxiaminoácido (84), (85), que era la L. (+) -serina, de fórmula $HOCH_2CH(NH_2)COOH$, que se une al grupo hidroxilo del radical fosfórico en forma de un eter del ácido fosfatídico.



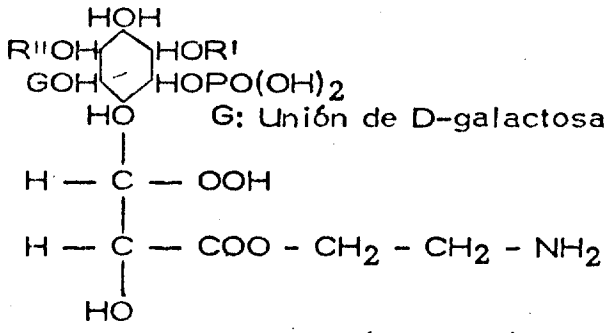
α-fosfatidilserina

Parece ser que la fosfatidiletanolamina es un producto de descomposición de la fosfatidilserina, por descarboxilación de ésta (140).

Tanto las fosfatidilcolinas como las tostatidiletanolaminas poseen grupos ácido y base que, muy probablemente se compensen, en tanto que en las fosfatidilserinas predomina el grupo ácido, por lo que sus reacciones químicas son propias de éste, y así, en productos naturales, se encuentran asociadas con iones potasio o sodio (3).

Fosfoinositoides

Un tercer grupo se viene a sumar a la mezcla conocida como "cefalinas". Los primeros en de detectar la presencia de ácido inositol-monofosfórico en el grupo de cefalinas de la soja fueron Klenk y Sakai (103) y posteriormente, por fraccionamiento de este grupo Folch y Woolley (86), (87), (89), (138) encontraron los fosfoinositoides en la fracción menos soluble, aislando Woolley (139) el compuesto que denominó LIPOSITOL:

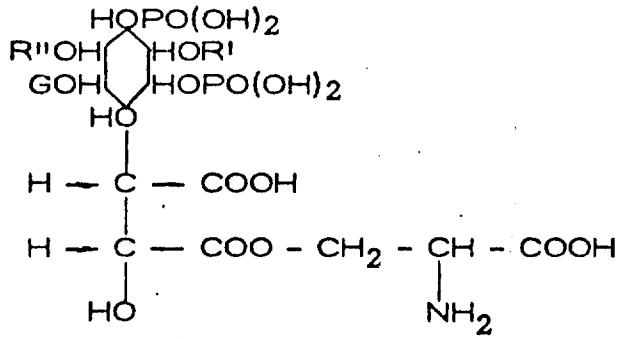


G: Unión de D-galactosa

- Monofosfonositoide (lipositol)

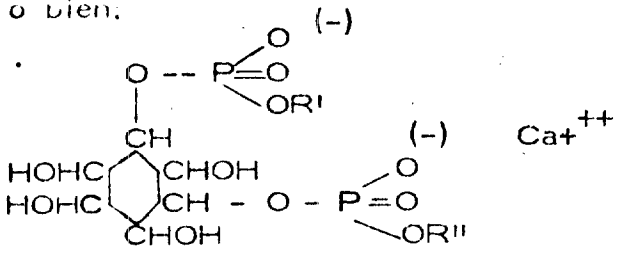
La hidrólisis total de este compuesto libera un mol de cada uno de los siguientes cuerpos: mesoinositol, galactosa, ácido fosfórico, etanolamina y ácido tartárico, junto con dos moléculas de ácidos grasos (3).

El difosfoinositoide fue aislado posteriormente por Folch (88):



Difosfoinositoide (10)

o bien:



Difosfoinositoide (3)

Ninguna de las estructuras de los dos compuestos indicados se conoce con certeza, pero se ha comprobado que estos compuestos figuran, en proporciones notables, en los fosfátidos, tanto de procedencia animal como vegetal (97), (123), (124), siendo su porcentaje del 40 % en los fosfátidos del haba de soja (127).

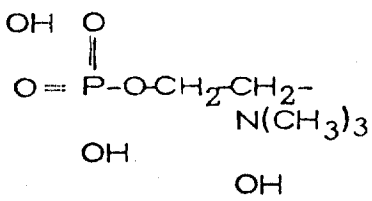
Esfingomielinas y cerebrosidos

En los fosfosfingósidos (10), también conocidos como esfingomielinas (11) la glicerina está reemplazada por la esfingosina

$$C_{13}H_{17}CH = CHCH(OH)CH(OH)CH_2 \\ NH_2$$

que satura uno de sus grupos OH con ácido fosfórico, que a su vez se esterifica con colina:

$$C_{13}H_{17}CH{=}CH{-}CH{-}CH{-}CH_2{-}NH{-}CO{-}R$$



Esfingomielina (10)

Las esfingomielinas, hasta el momento, sólo son de procedencia animal (12). En el caso de los galactosfingósidos no aparece el fósforo, y proceden de la esfingosina, uno de cuyos grupos hidroxilo está esterificado con la D-galactosa, mientras que el grupo amino se esterifica con ácidos grasos, como en la esfingomielina. Así ocurre en los cerebrósidos:

$$C_{13}H_{17}CH{=}CH{-}CH{-}\underset{\displaystyle OH}{CH}{-}CH_2{-}NH{-}CO{-}R$$

Cerebrósido $\quad OG$

Contenido en fosfátidos de las materias oleaginosas

Ha persistido durante bastante tiempo (17) la creencia de que los fosfátidos de origen animal eran diferentes de los de procedencia vegetal, siendo Levene y sus colaboradores los primeros que aislaron lecitina pura de la soja (105), (109) y trabajaron sobre los fosfátidos de esta semilla (106), (107), (108), a partir de cuyos trabajos se ha ido ampliando este estudio.

La cantidad de fosfátidos presentes en las diferentes semillas es generalmente bajo (no suele pasar del 0,5 %) y su porcentaje varía ampliamente, no sólo con el tipo de semilla, sino también con los métodos de extracción empleados por lo que en la literatura aparecen porcentajes bastante diferentes según los distintos investigadores, para una misma semilla.

Como indica Stanley (10) la proporción de fosfátidos en las semillas varía en relación directa con el contenido en proteínas en lugar del de aceite, siendo caso típico la soja (alto contenido en proteínas, alto contenido en fosfátidos) y la copra (alto contenido en aceite, bajo contenido en fosfátidos).

Contenido en fosfátidos de algunos aceites según Kaufmann (45)

Aceite	% Fosfátidos
Algodón	0,7 - 0,9
Arroz	0,5
Avena	1
Cacahuete	0,3 - 0,4
Cebada	3,4 - 4,2
Centeno	1,3 - 4
Colza	0,1
Linaza	0,3
Maiz	1 - 2
Mijo	0,2
Sésamo	0,1
Soja	1,1 - 3,2
Trigo	0,08 - 2,0

Lishkevich (111), (112), encontró que el algodón era la semilla que poseía

mayor porcentaje de fosfátidos, incluso superior a la soja, aunque las características de los fosfátidos de ambas semillas son muy similares (54), si bien los del algodón son algo más estables quizás debido a su menor índice de yodo ya que, según Thurman (65), (66), el índice de yodo de los fosfátidos comerciales de algodón es de 60 a 70, en tanto que para la soja llega a los 90.

Las cefalinas son los principales constituyentes de los fosfátidos de colza, maní (con 64,3 % de cefalinas y 35,7 % de lecitinas) y girasol (56), (con 61,5 % de cefalinas y 38,5 % de lecitinas).

Por otro lado, el aceite de sésamo (56), presenta el más alto porcentaje de lecitinas de todos los aceites de semillas (52,2 % de lecitinas y 40,6 % de cefátinas, siendo en el caso del áceite de linaza de 36,2 %.

El proceso de desgomado

Visto lo anterior, podríamos definir el desgomado como la serie de operaciones que preceden a la refinación (o que siguen a la extracción u obtención del aceite) y en las que las gomas o fosfátidos se separan antes e independientemente de la forma de eliminación de los ácidos grasos, tanto si estas gomas se destinan o no a la preparación de lecitina comercial.

Por consiguiente, básicamente, el proceso consiste en provocar la separación de las gomas, dentro de la masa de aceite, por la acción de agentes tales como agua, vapor, ácidos, etc.; aglutinar esos precipitados, y separarlos por diferencia de peso específico.

El producto separado de esta forma se compone de una variada serie de fosfátidos, algunos de ellos en muy pequeñas proporciones, así como otras substancias de naturaleza compleja y poco conocida, y de algunos restos de tipo sólido procedentes de las semillas extractadas, según el tipo de proceso. Dado el carácter graioso de estas mezclas se les da la denominación genérica de gomas, sin profundizar así en su composición.

Según la calidad de las gomas obtenidas y el proceso seguido en la separación de las mismas, se puede preparar a partir de ellas, por purificación y deshidratación, un producto comercial denominado Lecitina que, a pesar del nombre que recibe es básicamente una mezcla de fosfátidos (lecitinas, cefalinas, fosfátidos de inositol), aceite y ácidos grasos.

El desgomado no siempre se lleva a cabo como proceso separado o independiente de la refinación. En general se atiende al volumen de las gomas presentes en el aceite y a la posible utilización o comercialización del producto obtenido. Si el porcentaje de estos materiales en el aceite no es razonablemente notable, se someten directamente a refinación, y aún a veces, según el sistema que se emplee, aunque su porcentaje sea relativamente alto, se efinan también sin desgomado previo, englobándose las gomas en las pastas, como en el caso de la refinación alcalina.

En general, en los aceites de alto contenido en gomas, el desgomado se suele realizar casi siempre ya que facilita el proceso de refinación posterior y, en opinión de algunos investigadores y técnicos, reduce las pérdidas de refinación debidas al empleo directo de soda cáustica, dado la acción emulsificadora de los fosfátidos en solución alcalina, que aumentan el porcentaje de aceite ocluido con las pastas. También, a veces, un buen desgomado es suficiente para algunos aceites técnicos, como por ejemplo el aceite de linaza para barnices (1).
El efecto de los fosfátidos de varios aceites sobre las condiciones necesarias para la refinación alcalina del aceite crudo, ha sido estudiado por Fash (83).

La lecitina comercial, a pesar de las múltiples y variadas aplicaciones que se le han encontrado desde su aparición como subproducto de la

extracción del aceite, continúa siendo un engorro en las extractoras, ya que sólo un 10 a 30 %, según los casos, del total de lecitina obtenible en base a aceite crudo, tiene salida al mercado.

Sin embargo las necesidades del mercado pueden exigir, y la mayoría de las veces así ocurre, que aceites como soja, algodón o linaza, sean desgomados hasta un valor de contenido máximo en fosfátidos según los tipos y normas de comercialización.

Las gomas separadas del aceite (por hidratación la más de las veces), cuando no se destinan a su posterior tratamiento para la preparación de lecitina comercial, se suelen adicionar en estado húmedo a la harina extractada (normalmente mezclándola con ésta en el segundo o tercer piso del Desolventizador-Tostador, según diseños, y mejor en el Tostador, si se dispone de éste como aparato independiente). Esto representa un aumento del contenido graso de la harina, lo que no perjudica su valor alimenticio sino todo lo contrario, a la vez que ayuda a reducir el polvo que se desprende en el manejo de la harina.

Cuando la lecitina no se va a procesar, posteriormente a su obtención, es conveniente situar la centrífuga de desgomado y la bomba de gomas tan cerca del punto de utilización de las mismas como sea posible (DT u otro aprovechamiento cualquiera), para evitar los problemas derivados muchas veces de largos recorridos de tubería en la que la lecitina se solidifica o reseca, taponando el sistema. En cualquier caso, para minimizar estos problemas, es conveniente el trazado de vapor de la tubería de gomas, tanto húmedas como secas, así como una limpieza con agua caliente de la tubería cuando, por las razones que sea, el sistema se interrumpa durante largos períodos de tiempo.

La etapa de desgomado, independiente del proceso de refinación en sí, se suele llevar a cabo en las plantas de extracción de aceite, y como se apuntaba antes, la separación de los fosfátidos se suele realizar por hidratación y precipitación, aunque la eliminación nunca es completa. Por alguna razón de índole física no ha sido posible eliminar más de aproxima aproximadamente el 90 % de fosfátidos (52), pues aparentemente la adición de agua a estos compuestos no es completa.

El desgomado por hidratación

Es la forma generalmente más empleada para separar las gomas del aceite. Los fosfátidos, proteínas y otras impurezas de tipo coloidal son solubles en los aceites crudos secos, bien como soluciones verdaderas o bien en estado coloidal, pero cuando se adiciona agua a estos aceites, estas materias se hidratan y aumentan de peso, separándose de la masa de aceite en forma de coágulos fácilmente aglomerables y separables por decantación o centrifugación.

Las gomas, al aglomerarse, engloban en su seno un porcentaje de aceite que varía según las condiciones bajo las que se ha verificado la coagulación y separación Principalmente el porcentaje de agua empleado, así como la temperatura y la eficacia de la centrifugación, son los factores que parecen más directamente relacionados con este arrastre de aceite.

La molécula de un fosfátido presenta dos regiones de marcada diferenciación: una ocupada por las cadenas grasas (de los ácidos grasos), de carácter no polar, y la otra formada por el radical fosfórido y la base nitrogenada, que son de tipo polar. Estas dos diferentes tendencias de la molécula confieren a los fosfátidos un peculiar comportamiento físico-químico, tanto más acentuado cuanto mayor sea la distancia entre los anteriores grupos.

Los grupos polares se orientan hacia el agua, mientras las cadenas hidrocarbonadas huyen en ella,

creando una orientación molecular de formas paralelas. La región no polar atrae las cadenas hidrocarbonadas de las otras moléculas, aparentemente mediante fuerzas de Van der Waals (3) y se forma una segunda cadena de moléculas de fosfátidos tienen la misma tendencia hacia el agua, se van formando capas idénticas a las anteriores (77) en función al número de moléculas de agua.

Cuando los fosfátidos se encuentran en presencia de glicéridos (78) el caso es diferente, ya que las agrupaciones no estarán ahora formadas únicamente por agua y fosfátidos, sino que en las mismas entrarán también los glicéridos, La energía de interacción variará ahora de acuerdo con la proporción de fosfátidos y glicéridos.

Desnuelle (3), distingue dos máximos, el primero claramente diferenciado (a aproximadamente 70 % de fosfátidos y 30 % de glicéridos) y el segundo, menos preciso,aa unos 30 % de fosfátidos y 70 % de glicéridos. La formación de la capa mixta más estable corresponde, como era de esperar, a la máxima energía de interacción. Según Desnuelle, pues, "cualquier mixta formada espontáneamente en la superficie del agua debe por consiguiente contener aproximadamente 70 % de fosfátidos y 30 % de glicéridos". Así, cuando una solución diluida de fosfátidos en glicéridos se trata con una pequeña cantidad de agua, la masa separada, después de seca, contiene las proporciones anteriores.

La masa de fosfolípidos precipitados con agua está formada, según Jakubowski (41), por una doble monocapa de moléculas de agua rodeada por una monocapa de lípidos constituida por fosfolípidos y un 2-3 % de glicéridos cuando se hidrata aceite de soja fresco, o un 20-30 % de glicéridos cuando se hidrata aceite de soja que ya estaba parcialmente hidratado.

En su forma básica, las etapas implícitamente mencionadas de hidratación, centrifugado y secado, son las seguidas en la fabricación de la lecitina comercial a partir de los aceites crudos. Por consiguiente la aparición de glicéridos en la lecitina acabada no indica, a priori, una deficiencia del proceso o una pérdida por emulsificación, sino que gran parte de estos glicéridos han asistido, como parte activa, a la formación de las llamadas "figuras de hinchamiento" por lo que su presencia no se puede evitar, aunque su porcentaje es más o menos discutible o más o menos variable según las condiciones en que se verifican las dos primeras etapas mencionadas (hidratación y centrifugado).

Eficacia del desgomado por hidratación

No todas las gomas presentes en un aceite crudo parecen precipitables por agua, aunque el porcentaje de las que no lo son sea generalmente pequeño. Con arreglo a este carácter se han considerado dos grupos, de tipo general, denominados grupos hidrofílicos, al que pertenecen las gomas hidratables o precipitables por agua, y grupo ni hidrofílico, al que pertenecen las restantes. Mattikow (48) llama a las primeras "lipoides A" y a las segundas "lipoides B", estimando que en el caso del aceite crudo de soja el porcentaje de lipoides B es del orden del 5 al 10 %, precisándose para su separación, soluciones cáusticas fuertes, del orden de los 20°Bé.

Normalmente la cantidad de agua empleada en la hidratación varía de un 0,5 a un 3 %, determinándose casi siempre el porcentaje más adecuado mediante ensayos previos en laboratorio.

La cantidad de aceite que arrastran las gomas, independientemente del porcentaje más o menos fijo debido a la formación de las "figuras de hinchamiento", parece estar en una relación bastante estrecha con la cantidad de agua empleada, con la que también se relaciona la ordenación de capas fosfátido-agua-

glicérido, por lo que su influencia se acentúa más.

Dentro de ciertos límites, y para un mismo tipo de fosfátidos, a mayor porcentaje de agua empleado corresponde mayor volumen de gomas separadas, pero también las pérdidas de aceite, por arrastre, son mayores. Smith (59) considera que un tratamiento con 1 % de agua, o menos, producirá gomas que no disolverán más del 15 al 20 % de aceite, en tanto que Mattikow (48) estima que el aceite ocluído en las gomas es, aproximadamente, del 30 al 35 %, en base seca, si el porcentaje de agua adicionado es del 1,5 al 3 % y la separación se ha llevado a cabo por centrifugación.

En la precipitación de los fosfátidos la lecitina lo hace con 5 moléculas de agua por átomo de fósforo, mientras la cefalina emplea 40; en general los fosfátidos de soja precipitan con 7 a 9 moléculas de agua por átomo de fósforo (41).

La temperatura juega también un papel importante en la separación, quizás más relacionada directamente, con la centrifugación en sí (efecto sobre la diferencia en pesos específicos entre las gomas hidratadas y el aceite, así como sobre la viscosidad) que con la hidratación y coagulación propiamente dichas.

Como rango general, la temperatura de separación se sitúa entre los 55°C y los 82°C. En el caso del aceite de soja parece que la temperatura óptima de centrifugación está en los 75°C, apreciándose en el rendimiento una posible redisolución de parte de las gomas precipitadas por encima de dicha temperatura y un descenso en el rendimiento de separación a temperatura más bajas.

Burnasheva y Sterlin (28) observaron que calentando a 60°C soluciones de fosfátidos en aceite, disminuían las propiedades hidrofílicas de aquellos, siendo más acentuada esta disminución cuanto más alta era la temperatura. De ic.. forma la eliminación de fosfátidos por hidratación disminuía en el caso de soluciones en aceite que habían estado sometidas a calentamiento, siendo menor el rendimiento en fosfátidos separados cuanto más prolongado hubiera sido el calentamiento.

Desde el punto de vista del rendimiento en fosfátidos separadso entra en juego otro factor que es el tiempo de reacción o agitación durante la hidratación. Según Wittcoff este tiempo de agitación puede variar de 15 minutos a una hora (12), siendo normalmente de unos 30 minutos (42

Preparación de la lecitina comercial

El aceite crudo de soja es casi exclusivamente del que se utilizan las gomas precipitadas para la preparación de lecitina comercial, denominada comunmente "lecitina de soja".

Las gomas obtenidas a partir del aceite de algodón, dado su carácter resinoso y su aspecto oscuro, no tienen hasta el presente aplicación comercial por lo que, cuando se separan del aceite crudo, se suelen adicionar a la harina extractada.

Tampoco en el caso del aceite de linaza son aprovechables las gomas, aunque se practica el desgomado muy frecuentemente, sobre todo para la preparación de aceites técnicos. Con el fin de obtener un buen rendimiento de desgomado conviene ajustar lo más adecuadamente posible el porcentaje de agua de hidratación. Kantor (44) estima que el peso específico del aceite de linaza es función de su contenido en gomas, por lo que conocido aquel se puede fijar el porcentaje de agua de tratamiento. Este porcentaje suele variar de 0,2 a 2 % para pesos específicos de 0,9315 a 0,9340, respectivamente.

Un típico aceite de soja, según Bayley (11) rendirá aproximadamente un 3,5 % de material gomoso consistente en un 25 % de agua y un 75 % de productos solubles en aceite, de los que, aproximadamente, 1/3 es aceite y el resto insolubles

en acetona (básicamente fosfátidos). Como se ha dicho, prácticamente toda la lecitina comercial procede del aceite de soja, y precisamente, el extractado por disolventes, ya que las altas temperaturas empleadas en el prensado hidráulico o mediante expellers tienden a descomponer los fosfátidos (12).

Myers (53) ha patentado un sistema de desgomado y preparación de lecitina, si bien en general, estos procesos se puede considerar que responden al esquema representado en la Fig. 1

Con el fin de que la lecitina obtenida sea lo más clara posible, el aceite crudo procedente de extracción o de tanques de almacenamiento se somete a un filtrado previo a la hidratación, empleándose del orden de un 0,06 % de ayudante de filtración que se mezcla en el tanque de alimentación del filtro, que generalmente es de tipo horizontal y placas verticales con malla metálica.

Normalmente se suele emplear un solo filtro de capacidad calculada para manejar algo más de la producción diaria de aceite a tratar y, el volumen del depósito de recogida de aceite filtrado, así como el de mezcla de aceite y ayudante de filtración se calculan de forma que puedan contener algo más del aceite en tránsito que se maneja durante la limpieza del filtro que se efectúa una vez cada 24 h, con lo que el proceso resulta en continuo.

Se ajusta la temperatura del aceite y la proporción de agua, que una vez adicionada al aceite y homogeneizada la mezcla, se somete a un tiempo de retención, con agitación media, con el fin de facilitar la separación de las gomas en el seno del aceite. Este tiempo de contacto se estima, para el aceite de soja, de unos 20 a 30 minutos, y se prevé calculando el volumen del tanque de hidratación de acuerdo con la capacidad de la instalación.

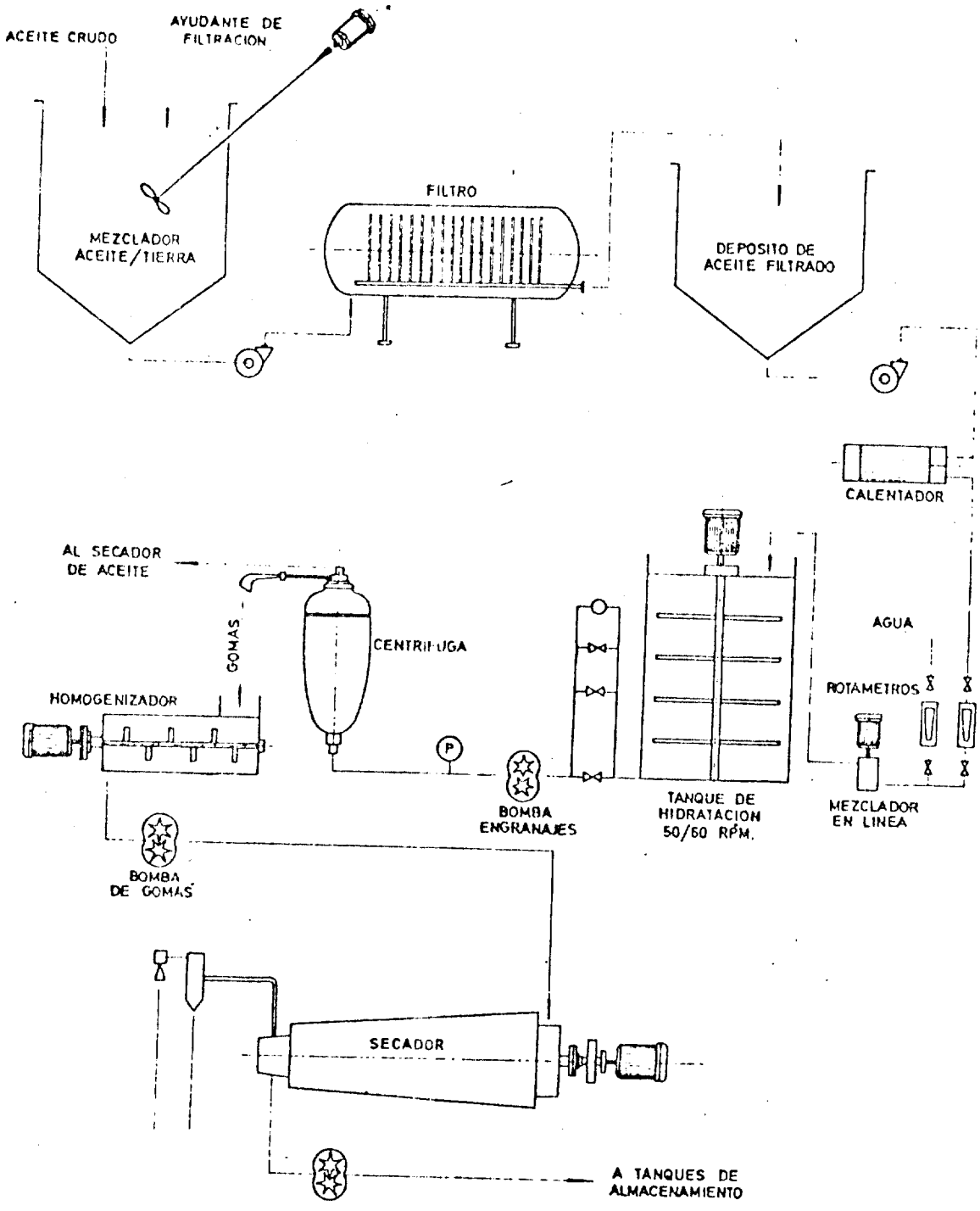
La salida del tanque de hidratación se suele hacer mediante una tubería en forma de lira o de U invertida, cuyo nivel superior corresponde al tiempo máximo de retención (o volumen máximo dentro del tanque) que se ha calculado según se indica anteriormente. Unas válvulas de comunicación entre las dos ramas de la tubería permiten variar el tiempo de contacto en función al nivel mantenido en el tanque de hidratación.

La mezcla de gomas y aceite se envía a las centrífugas, donde se efectúa la separación de las gomas y el aceite, el cual se envía al secador a vacío para eliminar el agua retenida y a continuación a los tanques de almacenamiento. Por su parte las gomas, después de homogeneizadas, se envían al secador de lecitina a vacío que, generalmente, suele ser del tipo de película fina que se lamina por la acción de dos troncos de cono coaxiales, con espesor de capa regulable y con caiefacción por camisa de vapor.

Jakubowski (98) da una descripción de una planta piloto de desgomado y Langhurst y Stanley (6) describen el proceso de preparación de lecitina comercial, que comienza por el tanque de deshidratación en el que las gomas se calientan y agitan a 65-71°C bajo unos 70 cm de vacío, hasta que su contenido en humedad se reduce por debajo de 0,5 %.

La lecitina seca, aproximadamente a 110-115°C, sale del secador de forma bastante fluída como para permitir su bombeo a los depósitos de recepción que, generalmente, están provistos de calentamiento eléctrico o por camisa de vapor, y tienen un agitador que permite homogeneizar la lecitina para el caso de que se adicionen substancias que modifiquen sus características físicas o químicas.

Uno de los factores que en principio condicionan la calidad de la lecitina obtenida, es su contenido en insolubles en benceno (BI), cuya fracción está constituida principalmente por "finos" de harina



DESGOMADO Y SECADO DE LECITINA

qué han pasado a través del filtro en la fase de clarificación previa. La cantidad máxima de BI permitida en la lecitina comercial es 0,3 %; sin embargo, si el porcentaje es sensiblemente superior a 0,1 % la lecitina aparecerá generalmente con un aspecto brumoso o nebuloso, en tanto que por debajo de dicho límite su aspecto será claro y brillante. Por otro lado parece que si el aceite sometido a hidratación es fresco y no ha sufrido un almacenamiento prolongado, la calidad de la lecitina obtenida mejora apreciablemente (11).

La lecitina seca obtenida por el procedimiento general descrito anteriormente, se conoce como lecitina comercial o lecitina cruda, y según Sullivan (62), empleando centrífugas herméticas con descarga a 3-4 k/cm^2 se puede obtener con valores tan altos como 80 % de insolubles en acetona (AI).

La riqueza en fosfátidos dependerá principalmente del método seguido para la precipitación y separación de las gomas, así como de del porcentaje de agua empleado, como ya se indicó anteriormente. Valores, por tanto, de 66 % de AI indicados por Bailey (11) o de 65 %, según datos de Stopper (61) son bastante frecuentes.

Las "lecitinas" comerciales no están formadas exclusivamente, ni siquiera principalmente, por este tipo de fosfátidos, sino que son una mezcla de lecitinas, cefalinas, fosfátidos de inositol, etc., así como ácidos grasos y aceite, que se cree ejerce una acción estabilizadora en estos productos tan sensibles a la humedad y el oxígeno (12).

La serie de compuestos que, en mayor o menor proporción, figuran en estas mezclas, podría ser aún más extensa si no fuera porque, en realidad, la extracción de los fosfátidos juntamente con el aceite, en los procesos continuos por disolventes, es bastante incompleta. La razón de esta deficiencia en la extracción de los fosfátidos se puede atribuir, principalmente, a dos factores: el tipo de disolvente empleado (siempre actúa más o menos selectivamente) y la humedad de la semilla, en forma de copos, que se somete a extracción, en la que la humedad se procura mantener lo más cerca posible del 11 %, lo que ejerce también una acción selectiva en el momento de la extracción.

Según Beckel y cbl. (25), el disolvente empleado en la extracción afecta no sólo a la acidez, sino también al contenido en gomas del aceite obtenido.

Efecto del disolvente de extracción sobre los valores de acidez y "rotura" del aceite de soja extractado (25)

Disolvente	Indice de acidez	"Rotura"
Dicloroetileno	0,52	-
Tricloroetileno	1,04	1,00
Tetracloruro de carbono	0,44	0,06
Isopropanol	0,95	0,86
Etanol	0,6	0,00
Etanol (residuo)	0,24	0,02
Hexano	0,50	0,50

El aceite de soja contenido en las gomas separadas por precipitación de este aceite, puede o no ser reemplazado por otro aceite más estable, tal como aceite de maní, coco, aceites hidrogenados o manteca de cacao (12). El proceso que se sigue en estos casos suele consistir en disolver el aceite en acetona, separar la solución formada y añadir el nuevo aceite.

También puede no añadirse aceite alguno y dejar los fosfátidos en estado más o menos libre, después de más de un lavado con acetona, pero el producto es muy inestable y debe almacenarse cuidadosamente con vistas a su pronto empleo.

Una técnica más económica que el empleo de acetona para la

purificación o concentración de los fosfátidos procedentes del desgomado de aceites, ha sido propuesta por Garmash (38) y consiste en disolven la "lecitina" en grasa hidrogenada a 95-100°C, centrifugando para eliminar las substancias insolubles a 80/85°C seguida de precipitación por enfriamiento a 40/45°C.

Las características físicas o químicas de la lecitina natural se pueden modificar mediante manipulaciones efectuadas durante su proceso de separación o posteriormente al mismo, como veremos a continuación

Decoloración de la lecitina

Aún bajo condiciones de proceso excelentes, la lecitina natural presenta un color parduzco, más o menos oscuro, cuyas tonalidades dependen principalmente de la variedad de semillas en proceso, así como de la calidad del aceite obtenido.

Con el fin de reducir este tono oscuro de la lecitina comercial natural, así como uniformar los tipos, independientemente de la fuente de origen de los aceites de los que se obtiene, se han propuesto varios agentes blanqueantes, entre los que destaca, por su profusión de empleo el peróxido de hidrógeno, que se añade a la emulsión de fosfátidos antes del secado, según una patente de Bollman (75).

Normalmente el peróxido de hidrógeno se suele añadir en el tanque de homogeneización de gomas, inmediatamente antes del secado, empleando una solución al 30-35%, en proporciones variables que pueden llegar hasta el 2 % con relación a los fosfátidos. El uso de este agente blanqueante se descrite también en patentes otorgadas a Bollman y a Bollman-Schwieger (74, 76), aduciendo que el peróxido de hidrógeno aumenta la capacidad de los fosfátidos para la emulsificación.

Según Thurman (67), cuando se emplea peróxido de hidrógeno como agente de [illegible]mante, los fosfátidos obtenidos tienen un color claro, producto de la decoloración efectuada en la fase de hidratación.

El uso de este agente blanqueante conduce al tipo de lecitina que se denomina "single bleached" (con blanqueamiento simple) y en ocasiones se refuerza su acción por el empleo de un segundo agente blanqueante, que generalmente es el peróxido de benzoilo, con lo que se origina el segundo tipo denominado "double bleached" (con blanqueamiento doble).

El empleo del peróxido de benzoilo como agente blanqueante también figura en la literatura (94, 126), en la que se indica que su utilización conduce a productos acabados de consistencia más suave.

Cuando se efectúa una decoloración doble de la lecitina se suele añadir al producto natural, aproximadamente 0,1 % de peróxido de hidrógeno comercial al 35 % (p. e. 1,132) y del orden del 0,008 % de peróxido de benzoilo seco, con relación al aceite en proceso de desgomado.

Se han propuesto también otros agentes químicos como el clorito sódico, según indica Greenfield (92), o peróxidos grasos como los propuestos por Epstein (88).

Entre los medios físicos de decoloración se han propuesto las arcillas y tierras de adsorción por Markman y Vuishnepolskaya (115), así como las tierras activadas por Marmor y Moyer (116), recomendando la decoloración en fase miscela, tomando el aceite en su fase intermedia de destilación, en el proceso de extracción por disolvente. Julian e Iveson (100) patentaron un procedimiento en el que la miscela del aceite de soja se trata con una arcilla de tipo ácido y luego se adiciona otra de tipo alcalino, aunque los fosfátidos se precipitan antes de la adición de la segunda.

Otras modificaciones de la lecitina natural

Además del color, la otra característica física que se modifica o se puede modificar, es la consistencia. Esta puede ser de naturaleza plástica, que es la forma en que normalmente se obtiene la lecitina natural, o fluida. Ambos tipos se diferencian en su contenido en ácidos grasos, y el paso de la primera forma a la segunda se hace por adición de un 2 a 5 % de mezcla de ácidos grasos a la forma plástica (11).

Según la composición de la lecitina obtenida por desgomado del aceite en proceso, se prepara el grado fluído en los mismos tanques de almacenamiento, por adición y mezcla de las proporciones convenientes de aceite de soja o ácidos grasos (generalmente también de soja).

En cuanto a las lecitinas químicamente modificadas, el tipo de lecitina dispersable en agua (denominado "water dispersible"), resulta de la adición y mezcla de un 8 a 10 % de un agente emulsificante y de un 4 a 5 % de Hexelene Glycol, a la lecitina fluida de blanqueamiento doble.

Las propiedades emulsificadoras de la lecitina cuyo contenido en aceite se incrementa al 30-40 % por adición de aceite de soja, se pueden aumentar por la adición de alcoholes aromáticos, propilgalato y estearato de polioxietileno (51).

Tipos de lecitina

Con arreglo a los tipos de modificaciones anteriormente descritos se distinguen seis tipos de lecitinas comerciales, según sean naturales, con blanqueamiento en cada una de sus dos versiones, plástica o fluída.

Caracte-rísticas	Plástica	Flúída
Humedad (a)	1 % máx	1 % máx
Insolubles en benceno	0,3% máx	0,3% máx
Insolubles en acetona	65 % mín	62 % mín
Indice de acidez	30 máx	32 máx
Viscosidad, poises a 25 25°C (b)	-	150 máx
Penetración (c (c)	22 mm máx	-

El color Gardner (en solución de aceite mineral al 5 %), es respectivamente de 10, 7 y 4 en valores máximos para las tres versiones: natural, con simple y con doble blanqueamiento, respectivamente.

a) Por destilación en tolueno durante dos horas o menos (AOCS, Ja 2-56)
b) Mediante un viscosímetro convencional adecuado o por el sistema de burbuja y tiempo de la AOCS, método Ka 6-59, suponiendo densidad uno. La lecitina fluida con viscosidad inferior a 75 poises se debe considerar sujeta a premio.
c) Utilizando el cono Precisión 13525, y el penetómetro 3510; con muestra acondicionada a 25°C durante 24 horas.

Continuará en el próximo número

Source: Inform Grasas Aceites
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DEGUMMING VEGETABLE OILS LECITHIN PRODUCTION*

by J. Lajara

Hydration using water vapor

Water for coagulation of phosphatides can be added to the oil as a vapor. In this case, one starts working with the oil at a temperature slightly over room temperature and the oil is heated up to 80°C using vapor. When the total moisture content of the oil is approximately 4%, centrifugation and separation of the precipitate is performed (68).

The basic problem with the use of vapor is the difficulty in measuring and controlling the percentage added and that is why in many cases hot water is used instead (1).

Vapor allows a better contact between the water and dissolved gums; nevertheless, the agitation stage is necessary in this case also.

Uses of commercial lecithin

The use of this sub-product of the refining of soy oil has increased significantly in recent years. Nevertheless, it is estimated that only 10 to 30% of the gums separated from the soy oil in the extractors go on the market as commercial lecithin since most of it is mixed with flours.

We would mention some of its uses: its emulsifying power and its natural origin make it especially useful in the food industry where it is used as a dispersing agent and to facilitate the mixture of fatty substances and dry flours or similar ingredients. It is also used as an additive in margarine, chocolate, etc.

It seems that phosphatides also present antioxidant properties (they are a natural antioxidant of the oils), although it is not very clear if its action is "per se" or if it only appears when they are associated with other compounds such as sterols or tocopherols. Sometimes they are used in small proportions to keep fats from oxidation and also in cosmetics and soap.

Its moistening and dispersing properties are also used industrially for the preparation of varnishes and inks.

The lubricants and the lead gasolines also benefit from its dispersing, emulsifying and antioxidant properties.

For more thorough information about "the industrial uses of the phosphatides", look up chapter XXVII, pages 504 to 522 of Wittcoff work (12) or D.R. de Castro (12A).

Other degumming methods

Besides water or water vapor as degumming agents, many other reagents or chemical compounds have been proposed that alone or two or one of them acting simultaneously, produce effects similar to hydration, sometimes completely; they can also affect later stages of the refining.

These specials reagents are used to obtain a better degumming process than the one achieved with water. In some cases they are intended to produce a partial or total neutralization of the oils or to achieve a purification that would make neutralization unnecessary.

Very few times are the gums precipitated by special reagents used for the manufacturing of commercial lecithin. The degumming is in this case more a refining stage than a finishing stage in the oil produced by extraction.

1. Use of alkalis -- the use of alkalis as degumming agents presupposes the desire to produce at least a partial neutralization of the oil. These are not recommended for the preparation of the lecithin since these reagents cause secondary effects in the purification of the oil, and these products are with the gums separated from the centrifugation.

a) Ammonium hydroxide

Ammonium hydroxide has been proposed by Clayton (32, 33) as a degumming and neutralizing agent. The gums obtained by this procedure used to be subjected to treatment to eliminate the ammonium and eventually recover it for further use.

Mattikow (49) gives a diagram of this system that generally corresponds to the one shown on page 38.

Generally, the oil is treated with ammonium-hydroxide (solution of NH_3 in H_2O) to separate the gums and at least part of the free fatty acids in the oil. When the acidity is low, the amount of ammonium hydroxide to be added corresponds stoichiometrically to the precise quantity needed to neutralize the free fatty acids, but in the case of higher acidities, the amount of reagent is lower than that necessary for a total neutralization.

Once precipitation of the gum has taken place, the heavy phase that precipitates in the centrifugue contains all the ammonia originally introduced into the system, as ammoniacal gums or ammonic soaps; also the excess of aqueous ammonia used, containing approximately 50% water.

With the purpose of helping the recovery of ammonia, the separated gums are diluted with approximately their weight in water, and the reagent is vaporized in an adequate container, heating the aqueous phase to the boiling point of the solution of ammonia/water at the system pressure. A further stage of exhaustion of the reagent followed by drying completes the cycle.

The oil once separated is free of gums and part of the fatty acids but contains a small amount of water and some ammonium hydroxide. If the oil is going to be refined once more it is not economical to recover the small amount of ammonium hydroxide, although if one wishes, it can be subjected to an exhaustion step as in the case of gums.

The concentration of the ammonium hydroxide can vary from 1.5% at the commercial concentration (approximately 29%) although the preferred values stand between 3 and 10%, so that there would not be too much fumes liberated during the centrifugation at 60-65°C.

The addition and mixture of the reagent is performed at 80-95°C when there is a further step of refrigeration; if not, it is performed at 65°C or a maximum of 80°C.

The quantity of reagent varies from 1 to 3.5% in weight of the oil and the amount of water to dilute the paste from 1 to 3 times the weight of the phase separated in the centrifuge. According to data from the patent, soy oil of 0.5% A.G.L. was treated with 1.5% of a solution of ammonium hydroxide (3.6%). The oil was heated at 65°C and the mixture at 80°C. After the centrifugation, the aqueous phase of the gums contained, in the dry base, 16.7% of the dragged oil.

b) Sodium hydroxide

The effect of the caustic soda, according to the concentrations used, on the gums of the oils, is more complete than the one achieved by simple hydration, and at the same time, its neutralizing action leads to a greater purification of the crude oils. For this reason, many times the oils are subjected directly to neutralization without degumming, especially in those cases in which the percentage of gums is reduced or partly reduced.

Nevertheless, the crude oils treated this way yield pastes in which the phosphatides have been attached and partially decomposed. That is why the use of great quantities of sulfluric acid is only to recover fatty acids of low quality and never as raw material in the manufacturing of lecithin.

In the refining of oils with caustic soda, before neutralization is finished, the phosphatides, which are more easily saponified than the neutral oil, start to decompose. For this reason when the acidity is calculated, the values are higher than for the same oil after degumming (42).

Use of acids

a) Phosphoric acid

It is the reagent used more frequently as a degumming agent when this stage precedes the addition of the base for the neutralization, in the refining processes, without separation of the gums independently of the soaps.

It is sometimes added as a concentrated solution (approximately 85%) and its percentage in relation to the weight of the oil seldom exceeds 0.1 %. Its coagulant and precipitating action on the gums is more pronounced than in the case of water, but due to its chemical action, it cannot be used unless it is followed by the addition of a neutralizing agent, before the centrifugation.

Because of this, it is not used as a precipitating agent of the gums in the preparation of lecithin; nevertheless, it is widely used to precipitate leftover gums present in oils previously degummed, or even crude oils, before the addition of caustic soda for the purpose of obtaining a more complete elimination of the gums and to reduce the absorption by the gums, once precipitated, of the reagent used in the neutralization (lower consumption of reagent) and neutral oil (lower losses). It seems that its use also improves the elimination of the soaps of the neutralized oil, in the washing stage.

The Farhenindustrie (82) process mentioned by Anderson (1) treats the soy oil at 35°C with 1% of phosphoric acid of a 40-65% concentration, heating the mixture at 60°C. After adding 0.2% of water and agitating during 10 minutes, the mass is left to deposit as a sediment and the precipitate is then separated, washing the oil with ammonium hydroxide to neutralize the remainder of the acid and precipitate any slimy residues.

The phosphorus contained in the linseed crude oils can be eliminated completely by treatment with a solution of phosphoric acid, it is interesting to point out that the phosphatides of the linseed oil have a higher content of phytoglycolipids, constituents isolated and studied by Carter (29).

Sullivan (63) proposes the addition of 0.1 to 0.4% of phosphoric acid until additional compounds are formed.

b) Nitric acid

Guillaumin and Boulet (39) studied different techniques for the degumming of oils controlling the effect of such treatments by the relation of the remaining phosphorous in the oil before and after the degumming.

Using diluted solutions of nitric acid (5%) at room temperature, they verified that 0.5-20% of the original phosphorous remains in the oil in the case of rape oil extracted by solvent, while in the one obtained by pressing, this percentage was 2%.

Above agitation speeds considered average there is redissolution of the gums (45% of the original phosphorus remains), while at lower speeds, the precipitation is incomplete (71% of phosphorus remains). At the average speeds of agitation, only 27% of the original phosphorus remains in the oil after the degumming.

Paul (55) considers this acid better than the hydrochloric or phosphoric acid for the precipitation of the gums.

c) Acetic anhydride

Hayes and Wolff (40) have described a process that uses acetic anydride anhydride as a degumming agent. It is the opinion of the authors that the use of anhydrides requires, for the same effect as the degumming, less quantity than the amount corresponding to the acids from which they originate. At the same time also the temperatures can go as high as 100°C. The lecithin obtained, once the acetic acid has been eliminated by drying the wet gums, seems to have the same characteristics as those obtained by the usual methods, while the oil, free from the gums, can be subjected to the refining process without, according to the authors, a neutralization stage being necessary.

The process consists, basically, in treating the crude oil with 0.1 to 1% of acetic anhydride, followed by stirring of the mixture during approximately 15 minutes and further addition of 1.5% of water, approximately, with more stirring for 30 minutes.

Following this, the mixture is centrifuged and the gums are separated, washing the degummed oil with 10% of water at 60-70°C to eliminate the acetic acid retained.

The system is adequate for peanut and soy oil, though in the case of cotton and corn it seems that the oils obtained cannot be bleached satisfactorily with dusts and carbons.

Myers (52) gives a further description of the method, with the data obtained.

d) Other acids

Other acids have been proposed as hydrating agents for the degumming of cotton oil such as solutions of weak boric acid (130, 132) or aliphatic polybasic acids (120).

Hydrochloric acid has been also proposed using approximately 0.4% of a 2N solution and completing the precipitating action with the addition of fibrous substances such as the flour from the extraction on the washed peels of the seeds.

Sulphuric acid (20, 72) is used primarily for whale oil, rape or fish (1) and for the preparation of oil of special technical characteristics. Its use requires special care to avoid sulfonation of the oil when the acid attacks the triglycerides, which produces a reddish coloration that cannot be eliminated.

In all pretreatments with acids or corrosive chemical products in general, it is important to take into account the capacity of the available equipment to make it possible to work under the operating conditions that are considered. Besides the probable deterioration of the equipment by chemical attack, the stability and color of the oil can suffer by the presence of iron salts or other metals, products of the attack on the containers and the tubing.

3. Various Reagents

Since water is the principal basis of the degumming reagent, different different variations exist. Acidified water (82) with a variable percentage of salt (60, 131) has appeared repeatedly in the technical literature.

Ayres and Clark (23) patented a process for the degumming of cotton oil that uses starch in water, and Sims et al. (58) in order to prevent development of an odor in its refined oil, proposed addition to the water used in the washing during the degumming process of 0.01 to 0.5% of an oxidizing agent such as potassium dichromate, potassium permanganate or

citric acid, operating at 50-70°C.

The technical literature also contains references to reagents such as alkaline phosphates (134), pyrophosphates (136), sodium hyposulfite (121), sodium sulfite (91) and moisturizing agents in general (90, 117, 119).

4. Various degummings according to the way they are performed

a) Degumming in the "micelle" phase

Among degumming processes in the micelle phase, Sikes (57) describes one performed experimentally with cotton oil. The oil, containing 8% hexane, was sent to the degumming stage without going through the finisher. The degumming was performed by the addition of a 2.5% of water, heating at 49°C (120°F) and centrifugation. After the centrifugating stage the oil was pumped to the finishing extractor and from there to the dryer to eliminate moisture. The results obtained seem to be comparable to the ones of the normal process, although Sikes points out an improvement in color, attributed, according to him, to the fact that the gums, due to the presence of hexane, absorb a great part of the gosypol contained in the oils, due to the greater solubility of this in hexane at the degumming temperatures. The gums, on the other hand, appear to be more difficult to handle in the laboratory.

Cavanagh and Beau (31) propose in the case of soy oil the following system: a micellar of soy oil containing 25-35% hexane is prepared and heated at 65-80°C. Two percent of water is added and the same percentage of ethyl alcohol, and the mixture is stirred until a precipitate appears that is separated by filtration. One percent water and 2% of ethanol are added until another precipitate appears and then the water and the solvents are eliminated at the same time or one after another. Instead of ethanol, methanol or isopropanol can also be used.

b) Degumming by absorption or filtration

It is known that a good bleaching with an adequate use of earths removes small percentages of gums retained in the oil after the degumming and neutralization steps.

When the oil has a low content in gums a mixture of 1% of activated soil can give a good result followed by the corresponding filtration after the neutralization stage. The effect of the activated soil is similar, though on a smaller scale, to that of the acids already seen, to which is added the absorption and retention power of the soil.

The advantage in these cases, besides the degumming action, resides in elimination of all solid particles that the oil can have suspended, this also being an advantage in the cleaning of the centrifuges of neutralization.

If the treatment with dusts is appropriate, the bleaching stage can be eliminated and the neutralized oil can be subjected directly to the deodorization at the same time that the recuperation of the neutralization would lead to fatty acids partially bleached.

Nevertheless in order for this treatment to be useful, from a degumming point of view, the percentage of gums of the crude oil must be substantially low.

c) Other methods

The same way that the bleaching acts as a separator and eliminator of the gums as well as deodorization produces, at the high temperatures of its operation, the precipitation of the slimy substances which coagulate due to the only effect of heat, above 240°C (1).

Nevertheless, the type of precipitate obtained is difficult to retain because the filtration engulfs to much oil in its mass.

As Anderson indicates (1) the action of an electric field of high voltage over the oil, can lead to the precipitation of the phosphatides, according to the processes proposed by Leinidorfer (104) and Unschuld(133).

Estimate of the contents of phosphatides in an oil:

The test most commonly used to determine how good the degumming of the oil was is the modified Gardner method of the AOCS ("Break test"), that basically consists of: the mixture is homogenized heating for 5 minutes at 65°C. 25 g are weighed and 3 drops of HCl are added, stirring well to facilitate the mixing.

A thermometer is placed with the bulb halfway in the liquid, without touching the walls and is heated up to 289°C without stirring and at an approximate speed of 75°/80°C per minute. It is cooled and 50 ml of CCl_4 are added, stirring to dissolve the oil. It is left to settle for an hour stirring every 15 minutes. It is filtered in a crucible with no less than 5 portions of 20 ml of CCl_4, breaking the vacuum at the end of each washing to make sure that the elimination of oil is complete. Following this the crucible is dried at 105°C to constant weight.

Break % = weight of the residue X 4

The phosphatide content corresponding to the precipitate obtained in this test is often approximately twice the value of the "break" obtained.

McGuire et al. (50) found that reduction in the nitrogen content is an indication of the efficiency of degumming. According to what Bauer (24) indicates, the nitrogen content of the soy oil extracted by solvent can vary between 0.019 and 0.023%

Given the fact that phosphorus is always part of the composition of the phosphatides, the determination of this element in the refined oils allows estimation of the residual percentage of them. According to Mattikow (47) the majority of the well refined vegetable oils have a percentage in phsophorus between 0.0005 and 0.0001% that is equivalent to 0.002 to 0.004% of phosphatides.

Nevertheless, it is risky to establish a direct relationship between the percentage of phosphorus and that of the phosphatides when there is not an absolute certainty about the original source of the phosphorus present that can be different than the phosphatides, and besides the molecular weight of the phosphatides is practically impossible to calculate (9).

The presence of the non-lipid phosphorus in the substances producing oil can be associated with components of different nature. As in the case of the soybean, according to Earle and Milner (79), a variety that contained 6.02 mg of phosphorus, for gram of whole seed showed the following distribution:

4% as inorganic phosphorus, 4% as nucleic acid

13% as phosphatide

79% as phytin

(Phytin has been described as the salt of hydrogen-calcium-magnesium or calcium-magnesium pottasium, of the inositolhexaphosphoric or phytic acid.)

According to Lishkevich (111) in the seeds of the oils investigated by him (cotton, soy, and peanut) only a 1.4 to 8.3% of the phosphorus present appears as phosphatide, while 52 to 76% appears as phytin.

To calculate the phosphatide content of a fatty extract or an oil, from the phosphorus content, a conversion factor is necessary, and this requires previous knowledge of the phosphorus content of the phosphatides but this is almost impossible due to the complexity of the mixture of these substances. Nevertheless, when in a fat or an oil there are no other substances containing oil but the phosphatides, the content in phosphorus is a direct indication of the amount of phosphatides present (64).

Many conversion factors had been proposed, deduced by experimentation and always using simplifications according to the method used. According to Jamieson and McKinney (43), the factor, based on the phosphorus content, of some phosphatides (such as the sphingomyelins) would appear to be 25.

An experimental factor of 22.7 was experimentally found by Artom and Fishman (69).

Due to limitations of the analytical methods with its errors, any of these factors would be,in principle,acceptable. In the case of the soy oil it seems to be right to use a factor of 30.

Influence of the phosphatides in the quality of the refined oil.

The presence of phosphatides in the oils can produce effects more or less noticable that many times are not clear.

As emulsifying agents they directly help in the formation of foam, that is not desirable in refined oils; at the same time also because of making the air solution more stable, they help the contact of oxygen with the oil and the absorption of moisture.

Kozin and Sitnikova (46) studied the variation of the acidity and peroxide indexes during storage, at different temperatures and relative humidities, of sunflower oil to which had been added 0.25, 0.5 and 0.75% of phosphatides. They reached the conclusion that phosphatides deteriorate the oil because of absorption of atmospheric moisture and increase the percentage of water in the oil at the same time they precipitate together with other substances, slimy products, increasing too the microrganic activity.

Bollmann (27) and Olcott (54) attributed antioxidant properties to the phosphatides, while Kochendorfer and Smith (101), found that the commercial phosphatides from the purified soy constituted antioxidants of variable activity. On the other hand only choline, among the products resulting from the hydrolysis of the phosphatides, show any antioxidant activity and even so to be significant, it must be found in significantly high proportions (35) and phosphoric acid, present in the phosphatide molecules and due to the fact that they take the Fe ions, are effective antioxidants (36).

It has been shown that purified lecithin, from the soy phosphatides, does not show the oxidation of the distilled fatty ester of olive oil (95) and is not as effective an antioxidant as carotene precursors (96).

Though the lecithins were found inactive, Olcott and Muattil (118) found that cephalins, seem to have a slight antioxidant action upon refined cotton oil that is clearly seen with the phosphatides mixtures, when the oxidation is accelerated by cobalt peroxides (81).

Desnuelle (3) indicates that cephalins, due to the acid character, belong to the acid antioxidants, and they always act together with other compounds, not showing, when they are alone, any antioxidant property.

It seems right to assume that the antioxidant effects observed in lecithins and cephealins are due to their continued action with other added substances such as citric or ascorbic acid (26), or other substances in the oil such as tocopherols (114, 128, 137), whose content is decreased; phosphatides in the refined oils are less valuable as antioxidants.

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Desgomado de aceites vegetales; Fabricación de lecitina

J. Lajara

Continuación del número anterior.

Hidratación mediante vapor de agua

El agua para la coagulación de los fosfátidos se puede adicionar también al aceite en forma de vapor. En estos casos se comienza a trabajar con el aceite a temperatura ligeramente superior a la ambiente, y se calienta el aceite hasta unos 80°C mediante vapor vivo. Cuando el contenido total de humedad del aceite es de aproximadamente un 4 % se procede a la centrifugación y separación del precipitado (68).

El principal inconveniente del empleo de vapor es la dificultad de medición y control del porcentaje adicionado, por lo que muchas veces se recurre al empleo de agua caliente (1).

El vapor proporciona un mejor contacto entre el agua y las gomas disueltas, sin embargo también es necesaria aquí la etapa de agitación mecánica.

Aplicaciones de la lecitina comercial

El uso de este subproducto de la refinación del aceite de soja ha aumentado sensiblemente en los últimos tiempos, habiéndose encontrado cada vez nuevas aplicaciones al mismo. Sin embargo se calcula que solamente un 10 a 30 % de las gomas separadas del aceite de soja en las extractoras, salen al mercado en forma de lecitina comercial, ya que la mayor parte se mezcla con la harina, pues si bien las aplicaciones son muy diversas, los porcentajes de empleo son bajos.

No obstante la variedad de aplicaciones exigiría todo un capítulo para indicar algo substancial de las mismas, en cuanto a su razón, porcentajes de empleo y efecto de los mismos por lo que mencionaremos, de forma general, las principales aplicaciones.

Su capacidad emulsificadora y su procedencia natural, la hacen especialmente apta para la industria alimenticia, donde se utiliza como agente dispersante y como ayudante de mezcla de substancias grasas y harinas secas o ingredientes similares. Se utiliza también como aditivo en margarinas, chocolate, etc.

Parece que también los fosfátidos presentan propiedades antioxidantes (de hecho son un antioxidante natural de los aceites), aunque sobre este punto no está aún demasiado claro si su acción es "per se" o se presenta solamente cuando se encuentran asociados con otros compuestos del tipo de esteroles o tocoferoles, por lo que a veces se utilizan en pequeñas proporciones para preservar de la oxidación algunas grasas, así como en cosméticos y jabones.

Sus propiedades de agentes humectantes y dispersantes se utilizan también industrialmente para la preparación de pinturas, barnices o tintas.

Los aceites lubrificantes y la gasolina de plomo también se benefician, según los tipos, de las propiedades dispersantes, emulsificantes y antioxidantes.

Para una información mas amplia sobre "usos industriales de los fosfátidos" consúltese el capítulo XXVII, págs. 504 a 522 de la obra de Wittcoff (12) o el trabajo de D.R. de Castro (12A).

Otros métodos de desgomado

Además del agua o el vapor de agua como agentes desgomantes, se han propuesto una serie de reactivos o compuestos químicos que aislados, o en acción conjunta de dos o más de ellos, producen efectos similares a los de hidratación, a veces más completos y otras veces con acciones de campo más amplio; que afecta incluso a etapas posteriores de refinación.

El fin que se persigue con estos reactivos especiales es siempre obtener un desgomado más a fondo que el que se consigue con el simple tratamiento por agua, en el que vimos que el rendimiento nunca es total, También, en algunos casos concretos, se intenta producir una neutralización parcial o definitiva de los aceites, o procurar una purificación que haga innecesaria esta etapa de neutralización.

Pocas veces, por no decir casi nunca, las gomas precipitadas mediante reactivos especiales se utilizan para la fabricación de lecitina comercial. El desgomado es aquí más una etapa de refinación que de acabado de aceite procedente de extracción.

1.- Empleo de Alcalis - El empleo de álcalis como agentes desgomantes presupone ya el deseo de producir, al menos, una neutralización parcial del aceite. Desde el punto de vista de la preparación de lecitina no son, en principio, recomendables, dado que estos reactivos originan unos efectos secundarios de purificación del aceite, de los que los productos obtenidos acompañan a las gomas cuando éstas se separan por centrifugación.

a) Hidróxido Amónico

El hidróxido amónico ha sido propuesto por Clayton (32), (33) como agente desgomante y neutralizante. Las gomas separadas por este procedimiento han de someterse a tratamiento para eliminar el amoníaco retenido, y eventualmente recuperarlo y reutilizarlo. Este sistema, del que Mattikow (49) da una reseña de una planta en proceso, responde al Esquema general de pág.

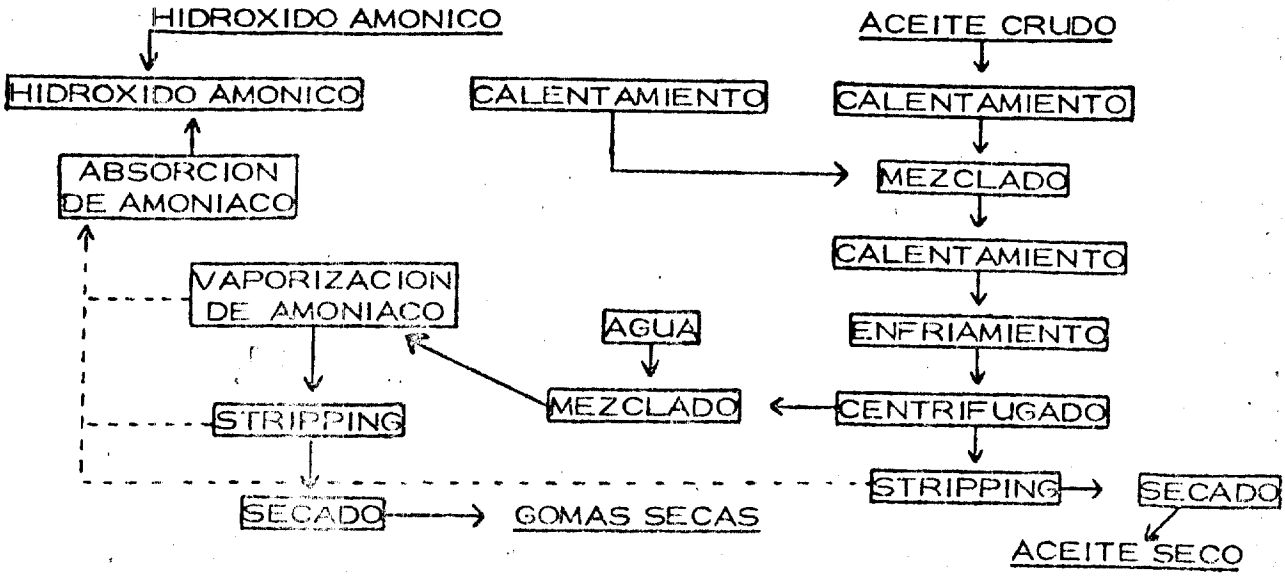
En líneas generales, el aceite se trata con hidróxido amónico (solución de amoníaco en agua) para separar las gomas y al menos una parte de los ácidos grasos libres del aceite. Cuando la acidez de éste es pequeña, la proporción de hidróxido amónico que se adiciona responde estequimétricamente a la cantidad precisa para neutralizar los ácidos grasos libres, pero en el caso de acideces mayores, la proporción de reactivo es menor de la necesaria para la neutralización total.

Producida la precipitación de las gomas, la fase pesada que se descarga en la centrífuga contienen substancialmente todo el amoníaco introducido originalmente en el sistema, en forma de gomas amoniacales y jabones amónicos, así como el exceso de amoníaco acuoso empleado, conteniendo aproximada aproximadamente un 50 % de agua.

Con el fin de favorecer la recuperación del amoníaco, las gomas separadas se diluyen con aproximadamente su peso en agua, y se vaporiza el reactivo en un recipiente adecuado, calentando la fase acuosa hasta el punto de ebullición de la solución amoníaco/agua a la presión del sistema. Una etapa posterior de agotamiento en reactivo seguida de secado, completa el ciclo de las gomas.

El aceite separado, libre de gomas y de parte de ácidos grasos, contiene una pequeña proporción de agua y con ella algo de hidróxido amónico. Si el aceite se va a volver a refinar no resulta económico recuperar la pequeña cantidad de

ESQUEMA GENERAL



hidróxido amónico que contiene, aunque si se desea se puede someter a una etapa de agotamiento del tipo de la empleada con las gomas.

La concentración del hidróxido amónico empleado puede variar desde 1,5 % a la concentración comercial (aprox. 29 %), aunque los valores preferidos parecen estar entre 3 y 10 %, procurando con ello que no haya abundante liberación de vapores al centrifugar a unos 60-65°C.

La adición y mezcla del reactivo se lleva a cabo a unos 80-95°C cuando hay etapa posterior de refrigeración, y en otro caso a 65°C o máximo 80°C.

La cantidad de reactivo varía de 1 a 3,5 % en peso de aceite y la cantidad de agua de dilución de pastas de 1 a 3 veces el peso de la fase pesada separada en la centrífuga.

Según datos de la patente, aceite de soja de 0,5 % A.G.L. se trató con 1,5 % de una solución de hidróxido amónico al 3,6 %. El aceite se calentó a 65°C y la mezcla a 80°C. Después de la centrifugación, la fase acuosa de gomas contenía, en base seca, 16,7 % de aceite arrastrado.

b) Hidróxido sódico

El efecto de la soda cáustica, según las concentraciones de empleo, sobre las gomas de los aceites, es más completo que el conseguido por la simple hidratación, a la vez que su acción neutralizante conduce a una mayor purificación de los aceites crudos. Por este motivo se someten muchas veces los aceites a neutralización directa, sin etapa previa de desgomado, principalmente en aquellos casos en que el porcentaje de gomas es reducido o medianamente reducido.

Sin embargo los aceites crudos tratados de esta forma originan pastas en las que los fosfátidos han sido atacados y parcialmente descompuestos, por lo que el tratamiento de las mismas, con abundantes cantidades de ácido sulfúrido, no se utilizan más que para la recuperación de ácidos grasos de baja calidad y nunca como materia prima para la fabricación de lecitina comercial.

En la refinación de aceites con soda cáustica, antes de que concluya la reacción de neutralización, comienzan a ser descompuestos los fosfátidos, que son más fácilmente saponificados que el aceite neutro. Por este motivo, cuando se valora la acidez de un aceite crudo se obtienen valores más altos que para el mismo aceite una vez desgomado (42).

2. - Empleo de Acidos

a) Acido fosfórico

Es el reactivo más empleado como agente desgomante, cuando esta etapa precede a la adición de la base de neutralización, en los procesos de refinación, sin separación de gomas independientemente de los jabones.

Se suele adicionar en forma de solución concentrada (aprox. 85 %) y su porcentaje, en relación al peso de aceite, no suele exceder del 0,1 %. Su acción coagulante y precipitante de las gomas es más pronunciada que la del agua, pero debido a su acción química, no se suele emplear si no va seguido de la adición de un agente neutralizante, antes de la centrifugación.

Debido a este hecho no se emplea como agente precipitante de gomas para la preparación de lecitina, sin embargo se utiliza ampliamente para precipitar los restos de gomas presentes en aceites previamente desgomados, o incluso crudos, antes de la adición de soda caústica, con el fin de lograr una más completa eliminación de las gomas y reducir la absorción por éstas, una vez precipitadas, del reactivo empleado en la neutralización (menor consumo de reactivo) y de aceite neutro (menores pérdidas). Parece, además, que su empleo favorece la eliminación de los jabones del aceite neutralizado, en la etapa de lavado.

El proceso de la Farbenindustrie (82) citado por Anderson (1) trata del aceite de soja a 35°C con un 1 % de ácido fosfórico de 40-65 % de concentradión, calentando a con continuación la mezcla a 60°C. Después de añadir un 0,2 % de agua y agitar durante unos 10 minutos, se deja sedimentar la masa y se separa el precipitado, lavando el aceite con hidróxido amónico para neutralizar los restos de ácido y precipitar los mucílagos residuales.

El fósforo contenido en los áceites crudos de linaza se puede eliminar completamente por tratamiento con una solución de ácido fosfórico, siendo interesante notar que los fosfátidos del aceite de linaza son los que presentan un contenido más alto en fitoglicolípidos, constituyente aislado y estudiado por Carter (29).

Sullivan (63) propone la adición de un 0,1-0,4 % de ácido fosfórico, hasta formación de los compuestos de adición.

b) Acido nítrico

Guillaumin y Boulot (39) estudiaron diferentes técnicas de desgomado de aceite de controlando el efecto de dichos tratamientos por la relación de fósforo remanente en el aceite antes y después del desgomado.

Empleando soluciones diluídas de ácido nítrico (5 %) a temperatura ambiente, comprobaron que un 0,5-20 % del fósforo original permanece en el aceite en el caso del aceite de colza extractado por disolvente, mientras que, para el obtenido por prensado este porcentaje era del 2 %.

Por encima de las velocidades de agitación consideradas medias hay redisolución de gomas (permanece un 45 % del fósforo original) mientras que, a velocidades bajas, precipitación es sensiblemente la incompleta (71 % de fósforo remanente). A dichas velocidades medias sólo un 27 % del fósforo original permanece en el aceite después del desgomado.

Paul (55) sitúa este ácido antes que el clorhídrico o fosfórico en cuanto a a su acción precipitante de gomas.

c) Anhídrido acético

Hayes y Wolff (40) han descrito un procedimiento que emplea anhídrido acético como agente desgomante. En opinión de los autores del trabajo el empleo de anhídridos requiere, para el mismo

efecto de desgomado, menor cantidad que la correspondiente al uso del ácido del que proceden, a la vez que la temperatura de trabajo se puede llevar a las proximidades de los 100°C. La lecitina obtenida, una vez eliminado el ácido acético por secado de las gomas húmedas, parece tener las mismas características que la obtenida por el procedimiento habitual, en tanto que el aceite, libre de gomas, puede someterse al proceso de refinación sin que, en opinión de los autores, sea necesaria la etapa de neutralización.

El proceso consiste, básicamente, en tratar el aceite crudo con un 0,1 a 1 % de anhídrido acético, seguido de agitación de la mezcla durante un tiempo aproximado de 15 minutos, y posterior adición de un 1,5 % de agua, aproximadamente, con nueva agitación durante unos 30 minutos. A continuación se centrifuga la mezcla y se separan las gomas, lavándose el aceite desgomado con un 10 % de agua a 60-70°C, para eliminar el ácido acético retenido.

El sistema es apto para aceites de soja y maní, aunque en el caso de algodón y maíz parece que los aceite aceites obtenidos no se pudieron decolorar satisfactoriamente con tierras y carbones.

Myers (52) hace una descripción posterior del método, con datos de resultados obtenidos.

d) Otros ácidos

Para el desgomado del aceite de algodón se han propuesto como agentes hidratantes soluciones de ácido bórico débil (130 , 132) o de ácidos alifáticos polibásicos (120).

El ácido clorhídrico se ha propuesto empleando aproximadamente 0,4 % de una solución 2N y complementando la acción precipitante con la adición de substancias fibrosas, tales como la misma harina de extracción o cáscaras de las semillas aceitosas molidas (1).

El ácido sulfúrico (20), (72) se emplea principalmente para aceites de ballena, colza o pescado (1) y casi siempre con vistas a la preparación de aceites de características técnicas especiales. Su aplicación exige especial cuidado para evitar la sulfonación del aceite al ser atacados los triglicéridos por el ácido, lo que origina una coloración rojiza que ya no se puede eliminar. Por ésto la temperatura, y en general las condiciones de operación, deben ser cuidadosamente conducidas.

En todos los pretratamientos con ácidos o con productos químicos corrosivos en general, es importante considerar la idoneidad del equipo disponible para trabajar con dichos compuestos bajo las condiciones de operación que se consideren. Aparte del posible deterioro del equipo por ataque químico, la estabilidad y color del aceite puede sufrir por la presencia de sales de hierro, u otros metales, procedentes del ataque de los recipientes y las tuberías.

3. - Reactivos varios. Siendo el agua la base principal del reactivo de desgomado, existen diferentes variantes. El agua acidificada (82) o con un mayor o menor porcentaje de sal (60 , 131) ha aparecido repetidas veces en la literatura técnica.

Ayres y Clark (23) patentaron un proceso de desgomado para aceite de algodón que emplea una dispersión de almidón en agua, y Sims y col. (58), con el fin de retardar la reversión del aroma del aceite de soja refinado, han propuesto la adición al agua de lavado, durante la etapa de desgomado, de un 0,01 a 0,5 % de un agente oxidante como dicromato potásico, permanganato potásico o ácido nítrico, operando a 50-70°C.

También se encuentran en la literatura técnica referencias a reactivos tales como los fosfatos alcalinos (134), pirofosfatos (136), hiposulfito sódico (121), sulfito

sódico (91) y agentes humectantes en general (90), (117), (119).

4. - Desgomados diversos en cuanto al modo

a) Desgomado en fase miscela

Entre los procedimientos de desgomado en fase miscela Sikes (57) describe el efectuado, en forma experimental, con aceite de algodón. El aceite, conteniendo un 8 % de hexano, se envió a la etapa de desgomado sin pasar por el acabador. El desgomado se realizó mediante adición de un 2,5 % de agua, calentamiento a 49 °C (120° C) y centrifugado. Después de la etapa de centrifugado, el aceite se bombeó al acabador de extracción y seguidamente al secador para eliminar la humedad. Los resultados obtenidos parecen ser comparables a los del proceso normal, si bien Sikes apunta una mejora de color, atribuida por él al hecho de que las gomas, debido a la presencia del hexano, absorben una buena parte del gosipol contenido en el aceite, dada la mayor solubilidad de éste hexano a las temperaturas de desgomado. Las gomas, por contra, resultan, al parecer, mas difíciles de manejar en el laboratorio.

Cavanagh y Bean (31) proponen en el caso del aceite de soja el siguiente sistema: Se prepara y calienta a 65-80°C una miscela de aceite de soja conteniendo un 25-35 % de hexano. Se adiciona un 2 % de agua y el mismo porcentaje de etanol, y se agita la mezcla hasta aparición de precipitado, que se separa por filtración. Se añade un 1 % de agua y un 2 % de etanol hasta nuevo precipitado, y luego se eliminan el agua y los disolventes, bien juntos o por separado. En lugar de etanol también se puede emplear metanol o isopropanol.

b) Desgomado por adsorción y filtración

Es sabido que una buena decoloración, con adecuado empleo de tierras, termina de agotar pequeños porcentajes de gomas retenidas en el aceite después de las etapas de desgomado y neutralización.

Cuando el aceite es de bajo contenido en gomas, puede dar buen resultado una mezcla de un 1 % de tierra activada, seguido de la correspondiente filtración, antes de la etapa de neutralización. El efecto de las tierras activadas es parecido, aunque en menor escala, al de los ácidos que vimos anteriormente, a cuya acción se suma el propio poder de adsorción y retención de las tierras.

La ventaja en estos casos, además de la acción desgomante, radica en la eliminación de todas las partículas sólidas que pueda llevar el aceite en suspensión, con la ventaja que reporta en cuanto a limpieza de la centrífuga de neutralización.

Si el tratamiento por tierra es adecuado, se puede a veces eliminar la etapa de decoloración y someter el aceite neutralizado directamente a la desodorización, a la vez que la recuperación de las pastas de neutralización conducirá ácidos grasos ya en parte decolorados.

Sin embargo, para que este tratamiento sea útil, en lo que al desgomado se refiere, el porcentaje de gomas del aceite crudo debe ser sustancialmente bajo.

c) Otros métodos

De igual forma que la decoloración actúa como separadora y eliminadora de gomas, también la desodorización produce, a las altas temperaturas de operación en que se realiza, la precipitación de las substancias mucilaginosas que coagulan, por el sólo efecto del calor, por encima de los 240°C (1).

Sin embargo el tipo de precipitado que se obtiene, por las anteriores causas, es difícil de retener, por filtración engloba mucho aceite en su masa.

Según indica Anderson (1) la acción de un campo eléctrico de alta

tensión sobre el aceite, puede conducir a la precipitación de los fosfátidos, según los procedimientos propuestos por Leimdörfer (104) y Umschuld (133).

Estimación del contenido en fosfátidos de un aceite

El ensayo más comunmente empleado para determinar la bondad del desgomado de un aceite es el método Gardner modificado, de la AOCS ("Break Test"), que básicamente consiste en lo siguiente: Se homogeiniza la mezcla, calentándola durante 5 minutos a 75°C. Se pesan 25 g y se le añaden 3 gotas de ClH, agitando bien para facilitar la mezcla. Se coloca un termómetro de forma que el bulbo esté en el centro del líquido, sin tocar las paredes, y se calienta hasta 289°C, sin agitar y a una velocidad aproximada de 75/80°C por minuto. Se enfría y se añaden 50 ml de Cl4C, agitando para disolver el aceite. Se deja reposar una hora agitando cada 15 minutos. Se filtra en crisol con no menos de 5 porciones de 20 ml de Cl4C, rompiendo el vacío al final de cada lavado para asegurar total eliminación del aceite. A continuación se seca el crisol a 105°C hasta peso constante:

$$\text{Rotura (Break) \%} = \text{Peso del residuo} \times 4$$

El contenido en fosfátidos correspondiente al precipitado obtenido con este ensayo suele ser de aproximadamente el doble del valor del "break" obtenido.

McGuire y col. (50) encontraron que la reducción del contenido en nitrógeno es una indicación de la eficacia del desgomado. Según indica Bauer (24) el contenido en nitrógeno de los aceites de soja extractados por disolvente puede variar entre 0,019 y 0,026 % mientras que en los obtenidos por prensado los porcentajes se encuentran entre 0,019 y 0,023 %.

Dado que el fósforo entra siempre en la composición de los fosfátidos, la determinación de este elemento en los aceites refinados permite estimar el porcentaje residual de aquellos. Según Mattikow (47) la mayoría de los aceites vegetales bien refinados presentan un contenido en fósforo comprendido entre 0,00005 y 0,0001 %, que equivale a un 0,002 a 0,004 % de fosfátidos.

Sin embargo es arriesgado establecer relaciones directas entre porcentaje de fósforo y porcentaje de fosfátidos cuando no existe absoluta certeza sobre la fuente de origen del fósforo presente, que puede ser distinta de los fosfátidos, además de que el peso molecular de éstos es prácticamente imposible de averiguar (9).

La presencia de fósforo no lípido en las materias oleaginosas puede estar asociado con componentes de naturaleza diversa. Así en el caso del haba de soja, según Earle y Milner (79), una variedad que contenía 6,02 miligramos de fósforo por gramo de semillas enteras, mostró la siguiente distribución:

- 4 % como fósforo inorgánico
- 4 % como ácido nucleico
- 13 % como fosfátido
- 79 % como fitina

(la fitina - phytin - se ha descrito como la sal de hidrógeno-calcio-magnesio, o calcio-magnesio-potasio, del ácido inositolhexafosfórico o ácido fítico).

Según Lishkevich (111) en los aceite aceites de semillas por él investigados (algodón, soja, lino y maní entre otros) sólo un 1,4 a 8,3 % del fósforo presente lo está como fosfátidos, mientras que de un 52 a 76 % está como fitina.

Para deducir el contenido en fosfátidos de un extracto graso o un aceite, a partir del contenido en fósforo, hace falta un factor de conversión, lo que exige el conocimiento previo del contenido en fósforo de los

fosfátidos, pero esto, dada la complejidad de las mezclas de estas sustancias, es prácticamente imposible. No obstante y con las oportunas salvedades, cuando en una grasa o aceite no existen compuestos que contengan fósforo otros de los fosfátidos, el contenido en fósforo es una indicación directa de la cantidad de fosfátidos presente (64).

Varios fac ores de conversión han sido propuestos, deducidos por experimentación y siempre empleando suposiciones y simplificaciones según el método empleado. Según Jamieson y McKinney (43) el factor, basado en el contenido en fósforo de algunos fosfátidos concretos (como la esfingomielina), resultaría ser de 25.

Un factor de 22,7 fue hallado experimentalmente por Artom y Fishman (69).

Dadas las limitaciones de los métodos de análisis, con sus fuentes de error insalvables, cualquiera de estos factores serían en principio aceptables. En el caso del aceite de soja parece acertado emplear un factor de 30.

Influencia de los fosfátidos sobre la calidad del aceite refinado

La presencia de fosfátidos en un aceite puede originar, directa o indirectamente, efectos más o menos notables, que muchas veces no están nada claros,

Como agentes emulsificantes, favorecen directamente la formación de espuma, lo cual no es de desear en aceites refinados, al mismo mismo tiempo que al hacer más estable la solución de aire, favorecen el contacto del oxígeno con el aceite y la absorción de humedad.

Kozin y Sitnikova (46) estudiaron la variación del índice de acidez e índice de peróxido durante el almacenamiento, a diferentes temperaturas y humedades relativas, de aceite de girasol al que se había adicionado 0,25, 0,5, y 0,75 % de fosfátidos, llegando a la conclusión de que los los fosfátidos deterioran el aceite al absorber humedad del aire e incrementar el porcentaje de agua en el aceite, a la vez que precipitan, junto con otras substancias mucilaginosas, aumentando también la actividad microorgánica.

Bollmann (27) y Olcott (54) atribuyeron a los fosfátidos propiedades antioxidantes, mientras que Kochendorfer y Smith (101), encontraron que los fosfátidos comerciales de soja purificados, constituían unos antioxidantes de actividad variable. Por otro lado sólo la colina, entre los productos resultantes de la hidrólisis de los fosfátidos, presenta alguna actividad antioxidantes, y aún así, para ser apreciable, ha de encontrarse en proporciones substancialmente altas (35), sin emgargo al ácido fosfórico se atribuye una acción secuestrante de los iones de hierro, lo que, dada su presencia en las moléculas de fosfátidos haría de éstos, indirectamente, unos antioxidantes efectivos (36).

Se ha comprobado que la lecitina purificada, a partir de fosfátidos de soja no retarda la oxidación de ésteres de ácidos grasos de oliva destilados (95), ni que es efectiva como antioxidante frente al caroteno (96).

Si bien las lecitinas se encontraron inactivas Olcott y Mattil (118) hallaron que el efecto de las cefalinas sobre el aceite de algodón refinado parece tener una ligera acción antioxidante, que se aprecia claramente, con las mezclas de fosfátidos cuando se acelera la oxidación por peróxidos de cobalto (81).

Desnuelle (3) indica que las cefalinas, por su carácter ácido, pertenecen a los antioxidantes ácidos y éstos actúan siempre en conjunción con otros compuestos, no mostrando, cuando están aislados, ninguna propiedad antioxidante. .

Parece pues acertado suponer que los efectos antioxidantes observados en lecitinas y cefalinas, se deben a la acción combinada con otras stancias bien adicionadas, como ácidos cítrico o ascórbico (26) o bien presentes en el aceite, como ciertos tocoferoles (114), (128), (137), que al ir disminuyendo su contenido en los aceites, a medida que progresa la refinación, hacen menos aconsejable la presencia de fosfátidos en los aceites refinados.

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Decomposition of Lecithin in Parenteral Fat Emulsions

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ABSTRACT

Aqueous solutions of alcohol-soluble phosphatide, synthetic lecithin and purified egg lecithin develop hemolytic and other toxic properties over a period of time at ordinary room temperature, or at an increased rate at 40°C. The toxicity of emulsions made with phosphatide as a stabilizer also increases with time. The evidence suggests that hydrolysis of synthetic lecithin proceeds first to give a hemolytic product, presumably lysolecithin. When liberation of both fatty acids from L-alpha-dimyristoyl lecithin was complete and the myristic acid was removed, no toxicity was shown by the aqueous phase, presumably glyceryl phosphorylcholine. Attempts to decrease the hydrolysis of lecithin solutions did not succeed. Although the use of lecithin phosphatide preparations as emulsifiers for intravenous fat may have limitations as to time and temperature of storage, there is no clear indication from these studies that any real hazard is involved from the standpoint of clinical use.

MUCH OF THE WORK on parenteral fat emulsions has involved the use of lecithin-phosphatide preparations as emulsion stabilizers (1-4). In our early attempts to produce fat emulsions suitable for intravenous feeding, work was done on a newly developed alcohol-soluble soybean phosphatide.[2] This amorphous material contains about 1.4% N and 3.2% P and contains predominantly lecithin rather than cephalin. The preparation proved nonpyrogenic in rabbits by the U.S.P. test (vol. XIV) and produced no pathological effects in rats as measured by the method of Geyer *et al.* (5).

Various investigators (6-8) have reported the spontaneous decomposition of lecithin, in some instances with simultaneous development of hemolysis. This hemolytic material is assumed to be lysolecithin arising from the hydrolysis of only one fatty acid from the lecithin molecule. Tests were therefore sought to determine the extent and possible hazards attending the hydrolysis of lecithin as used to stabilize parenteral fat emulsions. Studies of the stability of crude alcohol-soluble phosphatide were supplemented by studies of synthetic lecithin and highly purified egg lecithin.

The present work shows that the intravenous tolerance of mice to phosphatide solutions or oil-in-water emulsions stabilized with phosphatide decreases upon standing. However, because toxicity of lecithin develops slowly at normal temperatures, emulsions containing lecithin may be made which do not present unusual hazards. In any case, in view of its wide use in intravenous emulsions, the spontaneous hydrolysis of lecithin appeared to present problems which deserved study.

METHODS

Preparation of Emulsions and Solutions. All emulsions referred to in this paper contained 1% alcohol-soluble phosphatide (unless otherwise specified), 10 or 15% U.S.P. corn oil or olive oil, 5% U.S.P. dextrose and water. The components were premixed by high speed mechanical stirring and then transferred to a Cherry-Burrell Junior Viscolizer[3] which was operated at 3,000 psi for 30 minutes. The temperature of the emulsion during homogenization was kept below 50°C. The final containers were autoclaved at 121°C for 15 minutes.

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TABLE 1. TOXICITY DEVELOPMENT IN PHOSPHATIDE-STABILIZED EMULSIONS

Phosphatide, %	Temp., °C	Toxicity After Storage at Various Temperatures		
		0 mo.	2 mo.	5 mo.
0.5	4	0/5 at 2 ml	3/3 at 2 ml	
0.5*	4	0/6 at 1 ml		0/3 at 1 ml
	25	0/6 at 1 ml		0/6 at 1 ml
	40	0/6 at 1 ml		0/6 at 1 ml
1.0	40	0/5 at 2 ml	5/5 at 0.5 ml 0/5 at 0.25 ml	
1.0*	4	0/6 at 1 ml		0/3 at 1 ml
	25	0/6 at 1 ml		0/6 at 1 ml 0/3 at 0.5 ml
	40	0/6 at 1 ml		3/3 at 0.5 ml
1.5	25	0/5 at 2 ml	3/5 at 2 ml 1/5 at 1 ml	
	40	0/5 at 2 ml	3/3 at 1 ml 0/5 at 0.5 ml	

Toxicity data represent the ratio of mice killed to the number injected at given number of ml/20 gm mouse.

* Also contained 1% Domal 14, obtained from Emulsol Corp., Chicago, Ill.

All lecithin or phosphatide solutions were at concentrations of 1% and were made isotonic with salt or dextrose.

Mouse Toxicity Test. The acute intravenous toxicity test in mice was conducted as follows: three or more mice, 18–25 gm, were injected in the tail vein with 2 ml of the test solution/20 gm mouse. The injection was made in about 15 seconds and the animals were observed for at least 72 hours thereafter. If death occurred at this dose the level was progressively lowered until a dose was reached that did not cause death. Usually, levels below 0.5 ml were not tested. Both emulsions and phosphatide solutions were tested for increasing toxicity after storage at 40°C, room temperature and 4°C.

Qualitative Measurements of Hemolysis. *Mouse test (in vivo).* Mice which died within 5 minutes after intravenous injection were decapitated and bled into 5% U.S.P. dextrose. This mixture was then centrifuged and the supernatant fluid was observed for the presence of hemoglobin. Mice which survived longer than 5 minutes after injection were killed.

Dog test (in vivo). Dogs were injected intravenously with an aged solution of phosphatide. Following injection, a blood sample was drawn from the dog and mixed with 5% dextrose. This mixture was then centrifuged and the supernatant fluid observed for the presence of hemoglobin. The amount and rate of injection was comparable to that which a human subject might be expected to receive in the form of a fat emulsion, i. e. 20 ml/kg injected over a period of 1½ hours.

Human blood test (in vitro). A solution of phosphatide in 5% dextrose (stored 3 months at 40°C) was mixed with heparinized human blood in the following proportions: 0.5 ml phosphatide to 2.5 ml of blood and 0.25 ml phosphatide to 2.5 ml of blood. After standing 5 minutes at room temperature, the mixtures were centrifuged and the supernatant fluid observed for evidence of hemolysis. The first mentioned ratio of phosphatide solution to blood is in the approximate ratio which might exist in the blood stream following an average injection. We also tested for hemolysis at one half this level of phosphatide in blood.

Quantitative (in Vitro) Measurements of Hemolysis. Both dog whole blood and washed cells resuspended in isotonic saline were used to test the hemolytic effects of emulsions *in vitro*. Blood was collected using heparin as anticoagulant. To 2.5 ml of whole blood or suspended cells was added 0.5 ml of the emulsion and the mixture was then incubated at 37°C for 30 minutes. To this was added 3 ml isotonic saline and the cells were centrifuged down. All of the supernatant was carefully drawn off and the cells were washed with 3 ml isotonic saline and again spun down. The supernatant was removed and combined with the previous supernatant. The concentration of iron in the combined supernatant was determined by the method of Wong (9) modified for use with an Evelyn photoelectric colorimeter. The total volume of combined supernatants was calculated by determining by hematocrit the percentage of plasma from a mixture of 2.5 ml blood or suspended cells and 0.5 ml of emulsion incubated as before. The total iron obtained in the supernatant from the sample of blood or suspended cells and emulsion could then be calculated by multiplying the concentration of iron in the combined supernatants by the total volume of the supernatants.

The value for iron obtained as described above represents not only the iron released by hemolytic action of the emulsion, but also *a)* that small amount normally present in plasma, *b)* any released by hemolysis caused not by the emulsion but by mechanical handling of the blood, and *c)* iron present in the 0.5 ml of emulsion added. The total iron for *a)* and *b)* was determined by repeating the entire procedure but using only 2.5 ml whole blood or suspended cells as suitable blanks without adding the emulsion. The iron content of 0.5 ml emulsion was determined by the Wong method. The amounts of iron in items *a*, *b* and *c* subtracted from the total iron indicates the amount of iron released from hemoglobin of red cells by the hemolytic action of the emulsion. The hemoglobin equivalent was then calculated assuming the iron content of hemoglobin to be 0.335%. In order to calculate percentage hemolysis the total iron content of whole blood or the resuspended cells had to be determined. Finally, the percentage hemolysis represents that fraction of total blood iron which was released by the hemolytic action of the emulsion.

RESULTS

Alcohol-Soluble Lecithin. The acute toxicity to mice of emulsions stabilized with alcohol-soluble soybean phosphatide was found to increase upon standing (table 1). As seen in the table, toxicity developed faster as storage temperatures increased. The phosphatide alone in isotonic dextrose also became toxic more rapidly with increased temperature (table 2). This similar thermal lability of both the emul-

TABLE 2. EFFECT OF VARIOUS TREATMENTS ON TOXICITY DEVELOPMENT IN AQUEOUS SOLUTIONS OF ALCOHOL SOLUBLE PHOSPHATIDE

Initial Treatment	Toxicity After Storage at 40°C			
	2 mo.	3 mo.	4 mo.	5 mo.
Control 1	3/3 at 1 ml			
Control 2	0/3 at 2 ml	3/3 at 2 ml		
Adjustment with NH_4OH to:				
pH 6.8	3/3 at 2 ml	3/3 at 2 ml		
	0/3 at 1 ml	0/3 at 1 ml		
pH 6.7	0/3 at 2 ml	3/3 at 2 ml		
		2/3 at 1 ml		
pH 7.0	0/3 at 2 ml	3/3 at 2 ml		
		0/3 at 1 ml		
pH 7.8	0/3 at 2 ml	0/3 at 2 ml	1/3 at 2 ml	1/3 at 2 ml
Ethanolamine added to				
pH 9.7	1/3 at 2 ml	0/3 at 2 ml	3/3 at 2 ml	
pH 8.0	0/3 at 2 ml	3/3 at 2 ml		
		0/3 at 1 ml		
Amberlite IR-4B* resin added to:				
pH 7.5	3/3 at 1 ml			
Methyl glucamine added to:				
pH 7.3	3/3 at 1 ml			
Buffered with phosphate to:				
pH 7.5	0/3 at 2 ml	0/3 at 2 ml	0/3 at 2 ml	2/3 at 2 ml
pH 6.5	0/5 at 2 ml	2/3 at 2 ml	3/3 at 2 ml	
		0/3 at 1 ml	0/3 at 1 ml	
Hydrogenation of phosphatide	3/3 at 1 ml			

Toxicity data represent the ratio of mice killed to the number injected at given number of ml/20 gm mouse. All phosphatide solutions were made isotonic with dextrose or salt and contained 1% phosphatide. All freshly-prepared emulsions killed no mice at 2 ml.

* Obtained from Rohm & Haas Co. Philadelphia, Pa.

fier and the complete emulsion suggests that all or part of the toxicity shown by the emulsions was due to changes in the phosphatide. This does not, of course, exclude the possibility of other chemical or physical changes in emulsions which may have untoward physiological effects.

An increase in titratable acidity in the phosphatide solution, about 0.4 mEq of acid/gm of phosphatide, occurred on storage for 1 month at 40°C. Increased toxicity to mice developed. There was also a consistent drop in pH in the unbuffered solution. Such changes would be expected to accompany hydrolysis of the phosphatide.

An ether extract of an aged phosphatide solution yielded a highly acidic residue upon removal of the ether. The neutralization equivalent of the residue was considerably higher than the theoretical value of fatty acids expected from lecithin. Despite repeated attempts at purification of this residue the nature of the acidic groups remains unidentified.

Aged solutions of alcohol-soluble phosphatide caused hemolysis of mouse and dog blood *in vivo* and human blood *in vitro*. Freshly-prepared solutions did not exhibit such properties. These solutions became hemolytic, however, within 3 months storage at 40°C. Appreciable laking of human blood occurred in the presence of only one-half the amount of phosphatide that might reasonably be injected (see METHODS).

A solution of phosphatide that had been stored 3 months at 40°C was injected into a dog at a dose of 20 ml/kg for a period of 5 days. During the first 4 days, this solution was injected over a period of 1½ hours, while on the last day the rate was doubled. There was no significant change either in red cell count or hemoglobin content (Hellige Hemometer) of the blood despite the fact that the injections caused considerable hemolysis (qualitative *in vivo* tests). After 9 months storage at 40°C, this same solution was lethal to mice at 0.75 ml/20 gm mouse or, by projection, 37.5 ml/kg. We then injected the same dog as previously mentioned at a dose of 37.5 ml/kg over a period of 1½ hours. Although no overt symptoms of toxicity resulted from this injection, qualitative tests showed that *in vivo* hemolysis had occurred.

Blood samples drawn following the injection of aged lecithin fat emulsions are sufficiently turbid to completely mask any visual *in vivo* hemolysis which may occur. Thus *in vivo* studies are not revealing. Quantitative *in vitro* studies with dog blood on the comparative hemolytic properties of fresh and aged phosphatide fat emulsions are summarized in table 3. Aged emulsions are clearly more hemolytic *in vitro* to dog blood than freshly prepared ones. Circulating blood apparently offers considerable protection against the hemolytic agents

TABLE 3. CHANGE OF IN VITRO HEMOLYTIC ACTIVITY OF PHOSPHATIDE-STABILIZED EMULSION WITH AGE

Storage	Whole Blood		Washed Cells	
	Hemoglobin released	Hemolysis	Hemoglobin released	Hemolysis
	gm/100 ml	%	gm/100 ml	%
Fresh	0.027	0.15	0.15	0.77
6 mo. at 25°C	0.55	2.9		100
6 mo. at 40°C	0.45	2.5		100

Emulsion contained 10% olive oil, 1% phosphatide, 5% U.S.P. dextrose and 84% water. Dog blood was used.

produced in aged phosphatide emulsions. This is shown by the fact that complete hemolysis occurs only when washed red cells are incubated with the aged emulsions. We may note also that an aged phosphatide solution which was lethal intravenously in mice at 1 ml/20 gm body weight caused no deaths in mice when injected intraperitoneally at 4 ml/20 gm.

Fresh and old phosphatide solutions were analyzed for choline by the National Formulary Method (10) and for phosphorus by the method of Fiske and Subbarow (11). Both methods were modified to avoid the hydrolysis step so that only the free choline and phosphorus were determined. Identical trace amounts of free choline and phosphate were found in each. This is in agreement with the work of Baer and Kates (12) showing that alkaline hydrolysis of synthetic alpha-lecithin primarily yields free fatty acids and gylceryl-phosphoryl choline.

Various attempts were made to eliminate or retard the development of toxicity in aqueous solutions of the phosphatide. Solutions were autoclaved at 121°C for 15 minutes and stored at 40°C for observation. These solutions were made isotonic with either dextrose or NaCl at the time of preparation or were made isotonic with dextrose immediately before injection.

None of the following procedures prevented the eventual development of toxicity: adjustment to neutral pH, buffering at neutral pH, or hydrogenation of phosphatide using Raney-nickel catalyst. The results of these experiments are shown in table 2. We have found that neither evacuation of air in the headspace of the bottle nor the addition of 0.02% sodium hydrosulfite and 0.1% cysteine hydrochloride will prevent toxicity development in phosphatide solutions.

Synthetic Lecithin. Samples of synthetic L-alpha-dimyristoyl lecithin and L-alpha-distearoyl lecithin were obtained through the courtesy of Dr. Erich Baer (13). A 1% solution of the distearoyl lecithin was originally non-toxic at 2 ml/20 gm mouse. After standing for 3 months at 40°C, it became toxic and hemolytic (1 of 3 mice fatal at 1 ml/20 gm). A 1% solution of the dimyristoyl lecithin was non-toxic before autoclaving at 2 ml/20 gm mouse. After autoclaving, this solution became toxic and hemolytic (3 of 3 mice fatal at 1 ml/20 gm). After standing 5 months at 40°C, crystalline material precipitated out of the solution of L-alpha-dimyristoyl lecithin. The yield from 50 ml was 0.237 gm of crystals melting at 53–54°C. The theoretical yield and melting point for myristic acid were 0.236 gm and 54°C. The mixed melting point with authentic myristic acid was 53–54°C. The filtrate, theoretically containing only glycerylphosphoryl choline, was tested intravenously in mice and found to be non-toxic at 2 ml/20 gm mouse as expected.

Purified Egg Lecithin. A sample of highly purified egg lecithin was kindly furnished for this study by Dr. Mary Pangborn (14). A 1% solution of this material in isotonic saline proved initially non-toxic in mice at 2 ml/20 gm mouse. After standing 2 months at 40°C, this solution was lethal at 1 ml/20 gm mouse and extremely hemolytic at this dose.

After standing for 8 months at 40°C, crystals precipitated out of 75 ml of the egg lecithin solution. These crystals were filtered off, washed with water and dried to yield 0.233 gm. The neutralization equivalent of these crystals was found to average 284, the equivalent to stearic acid. Assuming that the lecithin is distearoyl lecithin, complete hydrolysis would yield 0.534 gm of stearic acid. Using this figure, we estimate that the solution was at least 43% hydrolyzed in 8 months at 40°C.

Nature of the Toxicity. The cause of death following injection of aged alcohol-soluble phosphatide solutions is not clear. In many cases, severe toxicity was recorded before hemolysis became noticeable. Aged solutions of phosphatide cause an extreme cardiac depression on the ventricle of the isolated rabbit heart. Distilled water itself did not kill mice when injected intravenously at 1.5 ml/20 gm.

but did cause severe hemolysis. In contrast, aged solutions of alcohol-soluble phosphatide, which were lethal at 0.5 ml/20 gm in mice, appeared to cause little or no hemolysis of red cells by qualitative tests.

The toxicity may in part be due to fatty acids liberated from the lecithin molecule upon hydrolysis. Individual fatty acids were emulsified in the presence of neutral fat and found to be toxic intravenously to mice in relatively small doses. However, the relation of fatty acid toxicity to the toxicity of aged phosphatide preparations is not clear.

DISCUSSION

Work both with aqueous phosphatide solutions and with phosphatide stabilized fat emulsions has clearly shown that hydrolysis can occur to yield toxic products. Although we have not established the nature of all of these degradation products, there is indirect evidence that formation of lysolecithin is involved. It is of interest to note that toxicity of synthetic L-alpha-lecithin increased much more rapidly than that of purified lecithin from egg yolk, which in turn appeared to increase more rapidly than the toxicity of commercial alcohol-soluble lecithin, a relatively crude material. The reason for this difference is not clear.

The intravenous mouse toxicity test used to screen both emulsifiers and fat emulsions is arbitrary, and may or may not have corresponding significance for other species. The basic procedure used in this test is similar to that used in official tests for toxicity of certain antibiotics (15). On equivalent body weight basis, an injection of 2 ml/20 gm mouse equals 5000 ml in a 50-kg human. The injection is made in a matter of seconds. Thus the test is rigorous as regards its effects on fluid balance as well as such effects as hemolysis of blood cells and depression of blood pressure. A somewhat similar test has been used extensively in these laboratories as a check on the nontoxicity of intravenous protein hydrolysates. In general, the LD_{50} of isotonic protein hydrolysates is somewhat less than 2 ml/20 gm mouse, whereas the LD_{50} for isotonic dextrose and saline is somewhat higher.

Creditor (16) has found that fat emulsions (stabilized with soybean phosphatides) cause increased mechanical fragility of dog and human red blood cells. It is possible that, in part, both the *in vivo* and *in vitro* hemolysis observed by us is due to such fragility. Thus, the red cells may have been ruptured by the mechanical stress put upon them during the drawing of the blood for the *in vivo* test and during the handling of the blood for the *in vitro* test. The fact remains, however, that as solutions or emulsions containing phosphatide become older their capacity to induce mechanical fragility or actual *in vivo* hemolysis of erythrocytes does increase. It is significant in this regard to note that not all emulsions cause increased hemolysis (as measured by our tests) as they become older. For example, a freshly-prepared emulsion containing 10% olive oil, 0.5% Tween 60,[4] 5% U.S.P. dextrose, and 84.5% water produced *in vitro* hemolysis amounting to 1.3% with whole dog blood. The same emulsion after 9 months storage at room temperature produced only 1.1% hemolysis.

There is little doubt that freshly made phosphatide emulsions may be used safely, as demonstrated by many clinical studies. Our data suggest, however, that injection of aged preparations may be hazardous. Some evidence of toxicity appeared in our studies before the various phosphatide preparations became frankly hemolytic. Thus a test for the lytic effect of any sample just prior to use might not suffice to show natant toxicity. As measured by the mouse toxicity test, there is evidence that the rate of phosphatide hydrolysis is not entirely predictable. Different lots of alcohol-soluble phosphatide may act differently. Although the need is a clear one, we have not as yet been able to devise a satisfactory dating test for the safety of aged preparations.

On the positive side one may point to the excellent emulsifying properties of lecithin, also its apparent stability even through sterilization by autoclaving. Lecithin has the further great advantage that it is a physiologic emulsifying agent, contributing important nutritive value of its own. It should be made clear that we have no evidence that toxicity develops with all phosphatide or lecithin solutions, nor can we conclude that the reactions cannot be prevented.

The synthesis of pure L-alpha-lecithins by Baer and Kates (12, 13) provided a milestone

[4] Polyoxyethylene sorbitan monostearate, Atlas Powder Co., Wilmington, Del.

in the chemistry of the phosphatides. Inclusion in our studies of various synthetic phosphatides from Dr. Baer, together with the highly purified egg phosphatide from Dr. Pangborn, enhances the general significance of the results. Nevertheless it should be clear that crude phosphatides may act differently as to rate of hydrolysis, particularly when used as an integral part of a fat emulsion.

The authors are indebted to Dr. L. W. Roth, Dr. L. R. Overby and Mr. M. Freifelder for their interest and technical assistance in certain phases of this investigation.

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STUDIES ON THE ANTIHÆMOLYTIC PROPERTIES OF LECITHIN AND CHOLESTEROL.

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In the previous report [Tsai and Lee, 1941] we have shown that the antihæmolytic function of the plasma is largely due to cholesterol. But since the antihæmolytic activity of cholesterol in the plasma as indirectly calculated can only account for about 40 per cent. of that of the whole fresh plasma, we were led to assume the existence of some other antihæmolytic substances or of some reinforcing factors acting on the antihæmolytic system of the plasma. In an attempt to elucidate this intricate problem we thought it more feasible to begin with an investigation on the various interacting factors in a simple artificial system containing cell-hæmolysin-antihæmolysin. Until more is learned regarding the antihæmolytic properties of cholesterol in such a simple medium it is not advisable to attempt to analyse the antihæmolytic mechanism of a highly complex system such as the plasma. We have incidentally observed that lecithin is hæmolytic itself, but may become antihæmolytic when mixed with saponin. Since lecithin and cholesterol are important blood constituents, their interaction in antihæmolytic behaviour should deserve our first attention.

Part I.—Experiments with Saponin as the Lytic Agent.

Part I. of the present report deals with experiments on the antihæmolytic properties of lecithin and cholesterol against saponin. In the course of developing the technique many conditions were found to be influential, and these must be carefully observed during experimentation. It is therefore deemed necessary first to make a brief description of them.

Methods.

Preparation of Aqueous Suspension of Cholesterol.—The oil emulsion preparation mentioned in the previous report [Tsai and Lee, 1941] does not fit our present purpose because the oil or emulsifying agent present

[1] Fellow of British Boxer Indemnity Fund Administration.

in the system may influence the hæmolytic and/or antihæmolytic action, thus rendering the interpretation of data difficult. On the other hand, an aqueous suspension of pure cholesterol can be prepared which yields consistent results, and this was therefore employed in all our experiments. We found that preliminary solution in alcohol, followed by addition of water, gives a fine and stable colloidal suspension, and that subsequent evaporation of alcohol by boiling does not destroy the antihæmolytic activity of cholesterol provided the solution is not alkaline. Our procedure finally adopted was as follows: Crystalline cholesterol (E. Merck) was first dissolved in 96 per cent. redistilled alcohol (2·5 mg. per ml.). Two ml. of the solution was then removed and mixed with 20 ml. of warm water (about 60° C.). The mixture formed a colloidal suspension. The alcohol was then evaporated off by boiling the mixture over a small open flame until the volume had been reduced to 6–7 ml. After cooling, the final volume was adjusted to 10 ml. by adding distilled water. The final concentration of cholesterol was thus 50 mg./100 ml. This stock cholesterol suspension would keep unchanged for 3 or more days. Nevertheless, we always prepared a fresh sample for each experiment on the same day.

In actual experiments only 0·1 ml. of the stock solution of cholesterol was used for each 3 ml. cell-hæmolysin system so that the concentration of cholesterol in the system was only 1·67 mg./100 ml. In such dilute solution its colloidal state as well as its antihæmolytic potency remained unchanged for a considerable period of time.

Preparation of Cell Suspension.—Dog's erythrocytes were employed in all the experiments. Na-citrate was used as an anticoagulant (5 mg. for each ml. blood). The cells were washed with physiological saline. Overwashing caused hæmolysis; in most cases slight hæmolysis usually began at the fifth or sixth washing, but in some rare cases it occurred even earlier. As a rule, the more washings were made the more liability of spontaneous hæmolysis. With low-resistant cells the results are often unsatisfactory. Hence our standard method of preparing cell suspension was to wash the cells 3 times with 3 volumes of 0·9 per cent. NaCl each time. Any sample that showed a trace of hæmolysis during the last washing was discarded. If no hæmolysis took place during this washing, the cells were considered to be suitable, and were then diluted with buffered saline to a 50 per cent. suspension and used immediately.

Preparation of Saponin and Lecithin Solutions.—The hæmolytic agent used in the present experiment was saponin. The stock solution was made to 0·1 per cent. in buffered saline and used within 3 days. Just before use it was diluted to 0·02 per cent. In most experiments commercial lecithin (purchased several years ago from E. Merck) was employed both as hæmolytic and antihæmolytic agents. Fresh material prepared in our own laboratory according to the method described in

Morse's *Applied Biochemistry* was used only occasionally as a check. In all cases the material was dissolved (to make 0·05 per cent.) in buffered saline just before use.

Determination of Antihæmolytic Unit of Cholesterol and Lecithin.— Unless otherwise specified, the cell-hæmolysin-antihæmolysin system in all experiments were all of total volume of 3 ml., containing 0·1 ml. (0·05 mg.) cholesterol or lecithin solution (or 0·1 ml. water in control sample), 0·06 ml. of 50 per cent. cell suspension, and variable amounts

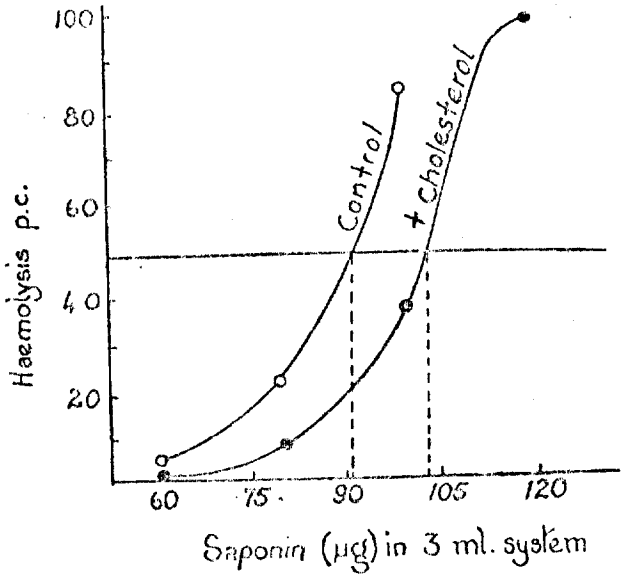

FIG. 1.—Graphs illustrating how the antihæmolytic potency of cholesterol is calculated. For explanation see the text.

of saponin and buffered saline. Saponin and saline were mixed first, followed by addition of cholesterol or lecithin. After thorough shaking, the cell suspension was then added. The mixtures were placed in a thermostatically controlled water-bath at 37° C. for 30 minutes. They were then centrifuged, and the degree of hæmolysis was determined by matching the test samples with the standards made from the same source of cell suspension. The antihæmolytic potency of 0·05 mg. of lecithin or cholesterol in the 3 ml. system was expressed in terms of micrograms (μg., or γ) of saponin inactivated by them. Fig. 1 illustrates our method of calculation. The control curve shows that to hæmolyse 50 per cent. of the cells in the 3 ml. volume requires 91·5 μg. of saponin. This quantity of saponin represents the cell resistance in μg. When 0·05 mg. of cholesterol is present in the system, the resultant curve shifts to the right side, showing 103·5 μg. saponin are now required for the production of 50 per cent. hæmolysis. The difference in the

quantity of saponin at the level of 50 per cent. hæmolysis is thus 12 μg., which is taken to represent the amount of saponin inactivated by 0·05 mg. cholesterol in 3 ml. That is the measure of the anti-hæmolytic potency of cholesterol. Similar calculation is applied to lecithin.

Cell Concentration.—The amount of saponin required for 50 per cent. hæmolysis increases with increasing concentration of the cells. However, as is shown in fig. 2, the relation of these two variables is

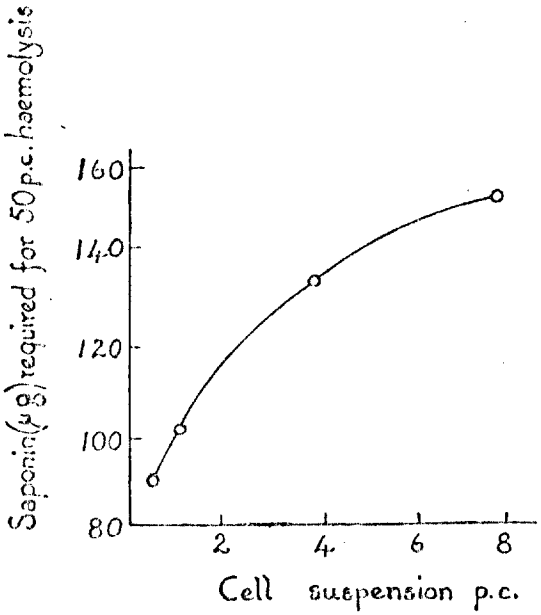

FIG. 2.—Relation of the amount of saponin required for 50 per cent. hæmolysis to cell concentration.

not linear. The milder degree of hæmolysis in higher cell concentrations may be due to the reduction of saponin concentration as a consequence of its greater dispersion or inactivation over a larger surface area of the cells. At any rate, for quantitative work cell concentration must be kept constant and uniform in different samples throughout all experiments.

RESULTS.

Hæmolytic and Antihæmolytic Action of Lecithin.—Lecithin is an auto-oxidizable and unstable substance. Consequently the results obtained vary somewhat with different samples of material. In general it is always hæmolytic when present alone with the red blood-cells, regardless of whether the sample of material is fresh or old. However, the hæmolytic potency increases with the age of the sample. This is

illustrated by the data in Table I., where, in column 4, larger numerical values signify lower hæmolytic potency and *vice versa*. When it is mixed with saponin it may remain hæmolytic or become antihæmolytic, depending upon the freshness of the material as well as its concentration. With fresh material its predominant action is still hæmolytic, whereas with old commercial preparation it is now pre-eminently antihæmolytic. This is illustrated by the data given in the last column of Table I.

TABLE I.—THE EFFECT OF AGE ON THE ACTIVITY OF LECITHIN.

Samples.	Date of preparation.	Date of experiment.	Lecithin, μg. required for 50 per cent. hæmolysis.	Saponin inactivated, μg.
1	Commercial	Jan. 14, 1941	1920	5
2	Commercial	Mar. 6, 1941	1800	5
3	Commercial	July 4, 1941	320	0
4	July 14, 1941	July 16, 1941	750	(−)* 9

* The negative sign (−) in this and following tables signifies hæmolytic. For the derivation of hæmolytic values see Part II.

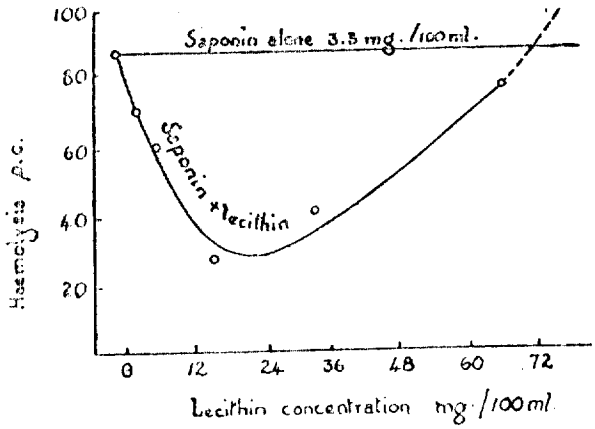

FIG. 3.—Relation of lecithin concentration in saponin solution to degree of hæmolysis. The reduction of hæmolysis at first increases with increasing concentrations up to a maximum and then gradually decreases as the lecithin content becomes higher and higher, until finally above a certain level no reduction of hæmolysis occurs. The dotted line is an extrapolation based upon the data from other experiments.

The antihæmolytic power of lecithin, when present, is rather weak, for it could never prevent hæmolysis completely, no matter what strength of it was used. When the concentration of saponin is fixed, the antihæmolytic potency of lecithin increases with its concentration

up to a certain maximum and then declines; it becomes hæmolytic again, when it is present in excess. This is shown in fig. 3. The

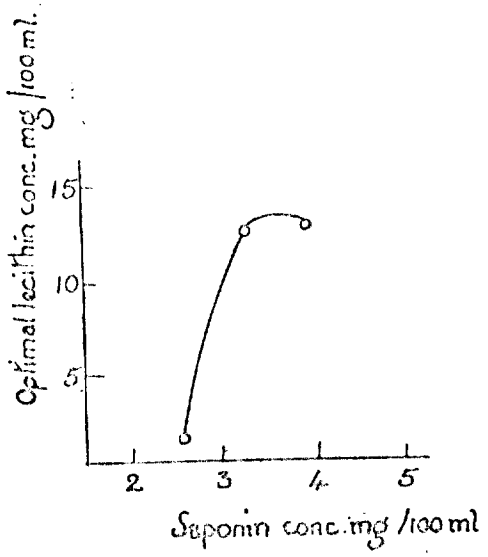

FIG. 4.—The relation of optimal lecithin concentration for antihæmolytic activity to saponin concentration.

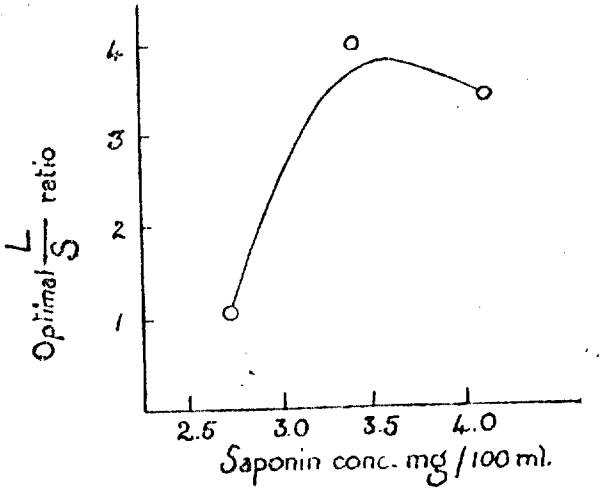

FIG. 5.—The relation of optimal $\frac{L}{S}$ ratio for antihæmolytic activity to saponin concentration.

extrapolation (dotted line) of the curve has been verified by a number of experiments and its validity cannot be doubted.

The optimal antihæmolytic power of lecithin varies not only with its own concentration, but also with that of the saponin. As illustrated by fig. 4, the optimal lecithin concentration for antihæmolytic activity increases rapidly with saponin concentration and soon reaches a maximum. With still higher saponin concentration the optimal lecithin content should be correspondingly increased. Nevertheless, the ratio of optimal concentration of these two substances is not constant; it increases up to a maximum and then declines (fig. 5).

Combined Action of Lecithin and Cholesterol against Saponin.—When both lecithin and cholesterol are present in the cell-saponin system reinforcement of antihæmolytic power is always observed regardless of the conditions of the material. It is greatest with fresh

TABLE II.—THE COMBINED ACTION OF LECITHIN AND CHOLESTEROL.

Cholesterol alone.		Lecithin alone.		Saponin inactivated by cholesterol + lecithin.		Increased inactivation of saponin, μg.
Amount in 3 ml., mg.	Saponin inactivated, μg.	Amount in 3 ml., mg.	Saponin inactivated, μg.	Calculated, μg.	Found, μg.	
0·05	12	0·05	5	17	101	84
0·05	12	0·20	33	45	287	242
0·05	12	0·50	83	95	317	222

samples and becomes weaker if the material has been exposed to air for some days. A summary of one typical experiment is given in Table II. It is seen that the antihæmolytic potency of these two substances when acting together is increased approximately 3 to 10 times. This reinforcement does not appear to rest upon the antihæmolytic action of lecithin and may involve an independent mechanism, because it occurs even in a concentration which would have been hæmolytic but for the presence of cholesterol. This is illustrated by the data from another sample presented in Table III. In other words, the reinforcing action of lecithin on cholesterol must be due to another mechanism independently of its antihæmolytic activity.

It is of interest to note that the degree of reinforcement varies with the $\frac{\text{lecithin}}{\text{cholesterol}}$ ratio in a given range of saponin concentration. As shown in fig. 6, which is plotted from the data in Table II., the optimal ratio seems to lie round 4 or thereabout. But this optimal ratio only applies to the range of saponin concentration of 3·3–10·6 mg. per cent. With an alteration of saponin concentration the optimal $\frac{L}{C}$

TABLE III.—THE REINFORCING ACTION OF LECITHIN ON CHOLESTEROL.

Cholesterol alone.		Lecithin alone.		Saponin inactivated by cholesterol + lecithin.		Increased inactivation of saponin, μg.
Amount in 3 ml., mg.	Saponin inactivated, μg.	Amount in 3 ml., mg.	Saponin equivalent, μg.	Calculated, μg.	Found, μg.	
0·05	12	0·05	0	12	160	148
0·05	12	0·10	(–)8	4	194	190
0·05	12	0·20	(–)8	4	180	176
0·05	12	0·50	strongly hæmolytic	strongly hæmolytic	90	> 90

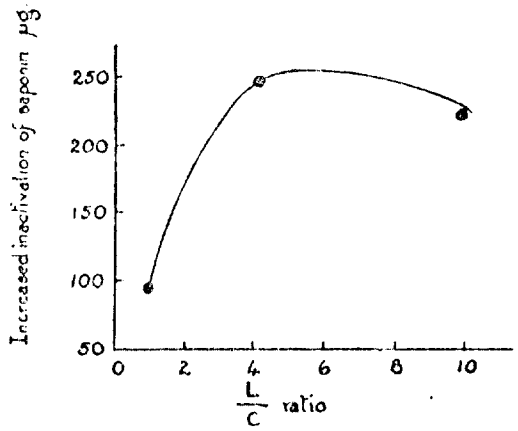

FIG. 6.—The relation of $\frac{L}{C}$ ratio to the degree of reinforcement of cholesterol action by lecithin, which is expressed in terms of an increase of the amount of saponin inactivated.

ratio also changes. The relation of these two factors has been studied in another experiment, the results of which are graphically represented in fig. 7. It is observed that the optimal ratio increases with saponin concentration, but the rate of increase is negatively accelerated.

Cholesterol is a hydrophobic colloid, while lecithin is a hydrophilic colloid; the former solution is known to be stabilized by the latter. It is quite possible that the reinforcement of antihæmolytic strength of cholesterol by lecithin may be due to this stabilizing effect. To test this hypothesis, experiments were carried out in which lecithin and cholesterol mixtures with and without saponin were allowed to incubate at 29° C. Table IV. contains the records of one of these experiments. When the three substances lecithin, cholesterol, and

saponin were incubated for 80 minutes (5), the antihæmolytic potency was higher than when incubated only for 10 minutes (3). This is not so if only lecithin and cholesterol without saponin (4) are incubated for similar length of time, indicating that reinforcement of cholesterol

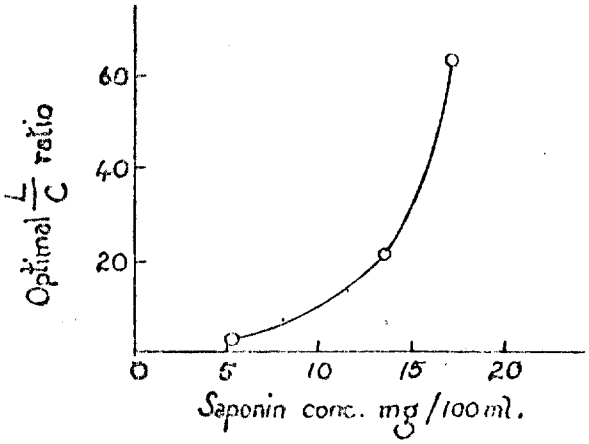

FIG. 7.—The relation of optimal $\frac{L}{C}$ ratio for antihæmolytic reinforcement to the concentration of saponin.

TABLE IV.—REINFORCEMENT OF CHOLESTEROL BY LECITHIN.

Substances.	Time of incubation, minutes.	Antihæmolytic potency in saponin inactivated, μg.
(1) Saponin alone	..	0
(2) Saponin + cholesterol . . .	10	12
(3) Lecithin + cholesterol + saponin .	10	125
(4) Lecithin + cholesterol. . .	70, then saponin added, further 10	102
(5) Lecithin + cholesterol + saponin .	80	165

action by lecithin takes place only in the presence of saponin. It is therefore very unlikely that the stabilizing action of lecithin can be the contributory factor in the reinforcement of cholesterol. Furthermore, as shown in the next section, lecithin is hæmolytic in the presence of cholesterol alone.

The Interaction of Lecithin and Cholesterol in a Lysin-free System.—In the preceding sections we have demonstrated that lecithin itself is hæmolytic and may remain hæmolytic or become antihæmolytic in the presence of saponin. It remains interesting to see whether the antihæmolytic effect of lecithin still occurs in the absence of saponin.

For the settlement of this point experiments were carried out in which saponin was omitted in the hæmolysin-antihæmolysin system—that is, lecithin was used as the hæmolytic agent and cholesterol as the antihæmolytic. In these cases lecithin remains hæmolytic and may be inhibited by cholesterol.

Discussion.

Levin [1935] has shown that pure fresh lecithin is antihæmolytic, whereas old commercial preparations are hæmolytic, especially in dilute solutions. Although the purity of our materials was not ascertained, our results are consistent, and differ from those of Levin in showing that fresh material is predominantly hæmolytic, whereas old commercial preparation may be either hæmolytic or antihæmolytic, depending upon its age and concentration. As lecithin is an auto-oxidizable and unstable substance, variation in its hæmolytic and antihæmolytic behaviour is to be anticipated. Nevertheless, we shall seek an early opportunity to repeat the above experiments with the crystalline substance.

Ponder [1934] has shown that when two lysins are present in the system the resultant action toward the cells may be mutual inhibition or acceleration. Thus, for instance, saponin + digitonin or + taurocholate are inhibitory and saponin + oleate and taurocholate + glycocholate are acceleratory. He postulated that the resultant action is due to the interaction of the two substances with cell constituents. The antihæmolytic action of lecithin against saponin hæmolysis reported in the present communication may be considered similarly as an outcome of interaction of these two lysins. As we shall describe in the next section, the behaviour of lecithin toward other lysins is not invariably antihæmolytic.

The most important fact demonstrated in this series of experiments is the reinforcement of the antihæmolytic activity of cholesterol by lecithin. In our previous paper we have emphasized the importance of cholesterol in the antihæmolytic action of the plasma and realized the complexity of the mechanism. We have now obtained evidence which leads to the suggestion that lecithin may also play an essential rôle in the protection of red blood-cells against the attack of lysins. One must, however, be aware that lecithin cannot prevent saponin hæmolysis completely without cholesterol. This indicates that it must act as an adjuvant of cholesterol in order to protect the cells with absolute certainty. As to how it behaves in the living body we do not know. Until we learn more about the conditions for the manifestation of reinforcement we must content ourselves without attempting further postulation and application.

SUMMARY.

1. Methods of preparing a simple artificial cell-hæmolysin-antihæmolysin system are described, and various influential factors reported.

2. Lecithin when allowed to act alone on the washed dog's red blood-cells is hæmolytic, but may remain hæmolytic or become antihæmolytic when saponin is present in the system, depending upon the freshness of the material and its concentration. With fresh material the reaction is predominantly hæmolytic, whereas with old commercial preparation it is eminently antihæmolytic, especially in dilute solutions.

3. The antihæmolytic activity of cholesterol is reinforced by lecithin (3–10 times). This reinforcement does not depend upon the antihæmolytic action of lecithin, and appears to be controlled by another mechanism. It is not due to the stabilizing action of lecithin on cholesterol solution.

4. The extent of reinforcement depends upon the ratio of $\frac{\text{lecithin}}{\text{cholesterol}}$ in a given range of saponin concentration. The ratio increases with an increase of saponin concentration up to a certain maximum and then declines.

5. Without saponin, lecithin remains hæmolytic in the cell-cholesterol system.

6. The antihæmolytic behaviour of lecithin toward saponin, and the reinforcing action on cholesterol, must be attributed to its interaction with saponin, because without the latter these actions cannot occur.

PART II.—EXPERIMENTS WITH OTHER LYTIC AGENTS.

In this part we desire to communicate some similar experiments on the antihæmolytic action of lecithin and cholesterol against other lytic agents. Lecithin used in this series of experiments was an old commercial preparation freshly removed from the stock bottle. The hæmolysins we have studied were digitonin, crude bile acid, sodium taurocholate, sodium glycocholate, oleic acid, and natural hæmolysin. The procedure of all the experiments was exactly the same as that described in Part I. Dog's citrated plasma or serum was employed as a natural hæmolysin against rabbit's washed blood-cells. In all other experiments washed dog cells were used. The antihæmolytic activity of a definite quantity of lecithin and cholesterol (unless otherwise specified it is usually 0·05 mg.) was expressed in terms of micrograms (μg.) of the lysin inactivated in the 3 ml. system. When lecithin was hæmolytic in the presence of another lytic agent its potency was expressed also in microgram equivalents of that particular lysin, but to distinguish it from that inactivated a negative sign (−) was added

in front of the numerical value. For example, in one experiment it required 50 μg. of oleic acid in the 3 ml. system to produce 50 per cent. hæmolysis of the cells when oleic acid was present alone. When this lysin was mixed with lecithin it required only 25 μg. oleic acid to cause the same percentage of hæmolysis. Hence the hæmolytic strength of lecithin in this particular case was equivalent to 25 μg. oleic acid. For convenience we wrote it as (–) 25.

Results.

Oleic Acid and Bile Salts.—Table V. gives the results of three typical experiments dealing with oleic acid, taurocholate, and glycocholate respectively. Lecithin appears to behave similarly toward these three

Table V.—The Effect of Cholesterol and Lecithin on Oleic Acid, Taurocholate, and Glycocholate Hæmolysis.

	Lysin equivalent or inactivated.	
	Found, μg.	Calculated, μg.
(1) Oleic alone	0	..
(2) Oleic acid + lecithin (0·1 mg.)	(–) 25	..
(3) Oleic acid + cholesterol	32	..
(4) Oleic acid + lecithin (0·1 mg.) + cholesterol	25	7
(1) Na-taurocholate alone	0	..
(2) Na-taurocholate + lecithin	(–) 160	..
(3) Na-taurocholate + cholesterol	440	..
(4) Na-taurocholate + lecithin + cholesterol	370	280
(1) Na-glycocholate alone	0	..
(2) Na-glycocholate + lecithin	(–) 245	..
(3) Na-glycocholate + cholesterol	400	..
(4) Na-glycocholate + lecithin + cholesterol	180	155

lysins in that it remains hæmolytic in all of them, though its hæmolytic power in each lysin system varies to some extent with different samples. With the presence of cholesterol the reinforcing effect of lecithin was observed in most cases. Thus, for instance, in the experiment presented in the first section of Table V. lecithin (0·1 mg.) has exercised a hæmolytic action equivalent to (–) 25 μg. oleic acid and cholesterol an antihæmolytic action equivalent to an inactivation of 32 μg. oleic acid. If the hæmolytic strength of lecithin remains unchanged in the presence of cholesterol, the sum of lecithin and cholesterol activities should be an inactivation of 7 μg. oleic acid. Since the observed value is 25 instead of 7, we assume that the hæmolytic action of lecithin is

reduced by cholesterol or the antihæmolytic power of the latter is reinforced by the former. Both these explanations are equally valid, although for the sake of consistency we prefer to adopt the latter. The same calculation and interpretation also apply to taurocholate, glycocholate, and other hæmolysins.

Crude Bile Acid Preparation.—This was prepared according to the method described in Mathews' *Physiological Chemistry* (1930). Its purity was not ascertained. But as its action differs from taurocholate and glycocholate, we thought it worth while to report our observation.

As shown in Table VI., crude bile acid preparation is hæmolytic. The behaviour of lecithin and lecithin + cholesterol toward it is similar

TABLE VI.—THE EFFECT OF CHOLESTEROL AND LECITHIN ON HÆMOLYSIS CAUSED BY CRUDE BILE ACID AND MIXTURE OF TAUROCHOLATE AND GLYCOCHOLATE.

	Lysin equivalent or inactivated.	
	Found, μg.	Calculated, μg.
(1) Crude bile acid alone	0	..
(2) Crude bile acid + lecithin	40	..
(3) Crude bile acid + cholesterol	20	..
(4) Crude bile acid + lecithin + cholesterol	75	60
(1) Taurocholate + glycocholate (1 : 3)	0	..
(2) Taurocholate + glycocholate + lecithin	(−) 300	..
(3) Taurocholate + glycocholate + cholesterol	500	..
(4) Taurocholate + glycocholate + lecithin + cholesterol	140	200

to that toward saponin, *i.e.* lecithin is antihæmolytic to crude bile acid and its antihæmolytic power in this particular case is greater than that of cholesterol. Furthermore, when lecithin and cholesterol are present in this lytic system they reinforce each other in antihæmolytic action.

Since lecithin is hæmolytic both in taurocholate and glycocholate, its antihæmolytic action toward crude bile acid must be due either to the impurity of the preparation or the interaction of taurocholate and glycocholate. To test the latter possibility several experiments were performed in which taurocholate and glycocholate in a proportion of 1 : 3 were mixed with lecithin, cholesterol, and lecithin + cholesterol respectively, and their resultant actions were determined as usual. The results from one of such experiments are incorporated in Table VI. It is obvious that lecithin is hæmolytic in taurocholate and glycocholate mixture, and it exerts practically no reinforcing action on cholesterol in such a system. Therefore its antihæmolytic behaviour toward crude

bile acid cannot be ascribed to the interaction of taurocholate and glycocholate, and must be due to some other unknown factor.

Digitonin.—Digitonin is known to be a strong hæmolytic agent. The behaviour of lecithin toward this lysin closely resembles that toward saponin; it is markedly antihæmolytic in digitonin and exercises a strong reinforcing action on cholesterol activity similar to saponin. This is shown in Table VII.

TABLE VII.—THE EFFECT OF CHOLESTEROL AND LECITHIN ON DIGITONIN HÆMOLYSIS.

	Digitonin inactivated.	
	Found, μg.	Calculated, μg.
Digitonin alone	0	..
Digitonin + cholesterol . . .	25	..
Digitonin + lecithin . . .	26	..
Digitonin + cholesterol + lecithin .	200	51

Natural Hæmolysin.—It has long been known since the work of Bordet that the serum of one animal may hæmolyse the washed red blood-cells of another animal of a different species. We have found that this type of hæmolysis can also be prevented by the plasma of the latter animal, by lecithin, and by cholesterol. The resistance of rabbit's

TABLE VIII.—THE EFFECT OF CHOLESTEROL AND LECITHIN ON HÆMOLYTIC SERUM.

	Serum inactivated.*	
	Found, μl.	Calculated, μl.
Serum alone	0	..
Serum + cholesterol (0·2 mg.) . .	40	..
Serum + lecithin (0·2 mg.) . .	40	..
Serum + cholesterol + lecithin . .	60	80

* The numerical values below are expressed in terms of microlitres of citrated plasma.

cells against dog's plasma or serum and the hæmolytic power of the latter vary with different individuals as well as many other conditions. Speaking generally, hæmolysis caused by 1 ml. of dog's citrated plasma can be completely prevented by 2 mg. cholesterol or 1·5–2·0 ml. of rabbit plasma. Lecithin is antihæmolytic to dog's plasma. The inactivation of the hæmolytic serum by lecithin, cholesterol, and these two substances together is shown in Table VIII., where the inactivation

is expressed in terms of microlitres (μl. or c.mm.) of serum. Here we see no reinforcement of cholesterol by lecithin.

The hæmolytic activity of the plasma is known to be due to the interaction of amboceptor and complement. Which of these elements is inactivated by lecithin and which by cholesterol? We shall report our experiments on this question in a separate communication.

Discussion.

Cholesterol is antihæmolytic to all chemical lysins studied. The significance of this finding should be emphasized. One might at first think that cholesterol acts on the cell membrane so as to protect it or prevent it from the invasion and attack of various lytic agents. But our results do not substantiate this hypothesis, for cholesterol is found to react with the lysins before the addition of the cells. In the second place, the degree of inactivation of different lysins varies. This is shown in Table IX., where the numerical values

Table IX.—Inactivation of Various Lysins by Cholesterol at Room Temperature.

	Amount required for 50 per cent. hæmolysis in 3 ml., μg.	Amount inactivated in 3 ml. by 0·05 mg. cholesterol, μg.	Percentages inactivated.
Digitonin	14	25	178
Lecithin	250	220	88
Oleic acid	45	27	60
Na-glycocholate	770	374	49
Na-taurocholate	880	380	43
Natural hæmolysin*	260	110	42
Saponin	90	12	13
Crude bile acid	220	20	9

* The numerical values in this row are expressed in terms of microlitres of citrated plasma.

were derived from different experiments of a similar nature. Although they are not strictly comparable, some rough idea may be gained from these computed data. If cholesterol acts on the cells alone, one should not expect such an extreme variation in the degree of inactivation of different lysins. We therefore believe that in many cases an interaction between cholesterol and the lytic agents must have taken place independently of the cells. If this proves to be true, then the possibility is chloresterol combines directly with the lysins. It seems unlikely it can combine with all of these chemically widely different substances.

While its antihæmolytic process against different lysins may differ in each case or in each group of substances, some may be chemical and some physical. Our experiments to be described in the following paper appear to verify this assumption.

Lecithin is antihæmolytic toward saponin, digitonin, crude bile acid, and natural hæmolysin, but remains hæmolytic in oleic acid, taurocholate, and glycocholate systems. In the first three substances of the former group the antihæmolytic action of cholesterol is reinforced by lecithin; the antihæmolytic activity and reinforcing action are most striking in saponin and digitonin. In the latter group the reinforcement is comparatively feeble and is manifested only by the reduction of the hæmolytic activity of lecithin. In other words, when both cholesterol and lecithin are present in a lytic system, the reinforcing effect occurs regardless of whether lecithin itself is hæmolytic or antihæmolytic toward that particular lysin originally. This is consistent with our statement that the reinforcement of cholesterol action by lecithin is independent of the antihæmolytic mechanism of the latter.

The antihæmolytic action of lecithin toward a number of lysins and its reinforcing effect on cholesterol have marked its importance in the antihæmolytic behaviour of the plasma. The antagonistic action of lecithin toward hæmolytic plasma suggests a likelihood of a similar function under normal condition in the plasma. It explains at least in part why we were unable in our previous investigation to account for the antihæmolytic potency of fresh plasma by the activity of cholesterol alone. We are certain that, besides lecithin and cholesterol, there are other substances and factors that share the antihæmolytic activity of the plasma, and hope that by similar methods of tackling the problem we may be able to explore the field more thoroughly and throw more light on the intricate mechanism under consideration.

Summary.

1. Lecithin is hæmolytic toward oleic acid, taurocholate, and glycocholate, but antihæmolytic to digitonin, crude bile acid preparation, and natural hæmolysin (dog's citrated plasma or serum against rabbit's corpuscles).

2. The antihæmolytic behaviour of lecithin toward crude bile acid is not due to the interaction between taurocholate and glycocholate.

3. The antihæmolytic activity of cholesterol is reinforced by lecithin in all lysin systems except natural hæmolysin, regardless of whether lecithin is inhibitory or acceleratory toward them in the absence of cholesterol. The reinforcement is most pronounced in saponin and digitonin. It is also very evident in crude bile acid and oleic acid. But it is rather feeble in Na-taurocholate and Na-glycocholate.

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CHOLINE AS A STIMULANT OF GASTRIC SECRETION

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This work was carried out as a contribution to the study of the second, or "chemical," phase of gastric secretion. The identity of the secretagogue bodies of the food responsible for the second phase is still unknown. The non-specific hydrolytic products of the three main classes of foodstuffs can play no important part: thus the amino acids stimulate gastric secretion either weakly, or not at all (1, 2); the fatty acids excite only after producing a marked inhibitory action; while the sugars are inactive. The extractive substances of foods, especially foods of animal origin, do, however, include bodies of high secretagogue potency, and Krimberg and Komarov (3) have pointed out that among these the nitrogenous bases are probably of especial importance. Campbell (see Babkin, 4), whose results were confirmed by MacIntosh (unpublished), found that practically the entire activity of fish-muscle extracts could be recovered in the fraction of the nitrogenous bases. Further fractionation by the silver-baryta method showed that the activity was due to the "arginine" and "lysine" fractions, the "purine" and "histidine" fractions being ineffective. Of the known compounds present, nearly all can be eliminated: thus histamine does not act from the digestive tract except in massive doses; adenosine is destroyed during tissue autolysis and by digestion; while creatine, carnosine, carnitine, and methylguanidine are too feebly active to be of any importance. Choline, on the other hand, is distributed widely in foodstuffs, and possesses well-marked physiological activity. It has been shown by Ivy and Javois (5) to excite gastric secretion on being introduced into the digestive tract, but no detailed analysis of its action has been made. We have therefore attempted such an analysis, with the aim of determining whether or not the secretagogue activity of choline is sufficient to account, on the basis of the choline content of various foods, for any part of the observed secretory responses of the stomach to these foods.

The effect of choline on the gastric glands is of further importance, since it is desirable to know whether the second phase involves the active participation of the peptic cells. This question has been discussed by Babkin (4). These elements are under the control of the vagus, and choline, since it exhibits parasympathomimetic activity, might be expected to stimulate them.

METHODS. Most of the experiments were carried out on three dogs equipped with pouches of the stomach, as follows:

"P": female, weight 14 kgm., equipped with a Pavlov pouch (vagal and sympathetic innervation intact) and a gastric fistula.

"H": female, weight 21 kgm., equipped with a Heidenhain pouch (sympathetic innervation only).

"B": male, weight 19 kgm., equipped with a Bickel pouch (extrinsic denervation as complete as possible without severing of blood vessels) and a gastric fistula.

The two last-named were used in most of the experiments, since their responses were not complicated by psychical factors, even when food was taken by mouth. When dog "P" was used, test-meals were injected directly into the stomach by gastric fistula; this procedure was also used with "B" when the test-meal was meat. The animals were all kept in good health over a long period. They received a uniform diet of lean beef heart, oatmeal, milk, and salt. All experiments were begun in the morning while the gastric glands were at rest, water having been previously given *ad lib.* Experiments were never begun if the resting secretion exceeded 0.5 cc. for 30 minutes: the daily secretion of the pouch when no food was given did not exceed 3 to 4 cc., practically all mucus.

The test-meals used were minced lean beef in quantities of 300, 500, or 700 grams, unsalted butter (100 grams) with 100 cc. water, and white bread (250 grams) with 200 cc. water. For comparable experiments the same lot of minced meat was used, since different lots vary rather widely in secretagogue potency: control experiments showed that the secretory effect of such meat was not changed by a week's storage in the ice-box. Choline was given as choline chloride (Merck). The lecithin used was a purified preparation supplied by the Department of Biochemistry, McGill University. On the day before each experiment, the animal received the standard meat meal. Experiments were not performed on successive days.

The pouch secretion was collected in the usual way and measured at half-hourly or hourly intervals. The volume of visible mucus in the juice was estimated separately. Free acidity and total acidity were determined by titration with Töpfer's reagent and phenolphthalein respectively. Pepsin was determined by Nirenstein and Schiff's (6) modification of Mett's method, the digestibility of the coagulated egg-white in the Mett's tubes being checked by means of a standard synthetic gastric juice freshly prepared from a commercial pepsin.

Acute experiments, designed to test more exactly the action of choline on the peptic cells, were carried out on dogs anesthetized with a chloralose-urethane (1:10) mixture. Both vagi were cut in the neck, the pylorus was tied, a small silver fistula was sewn into the ventral wall of the stomach, and the stomach was washed out with warm tap-w Hista-

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mine dihydrochloride (0.1–0.3 mgm. per kgm.) was injected subcutaneously at half-hourly or hourly intervals, and gastric juice for analysis collected during 15-minute periods. When a copious secretion had been established, choline chloride (5–20 mgm. per kgm.) was injected intravenously and the collection of juice continued. Acidity and pepsin were determined as above.

RESULTS. *Effect of choline injected intravenously.* Choline chloride (5 to 15 mgm. per kgm.) injected intravenously during the rest of the gastric glands, evoked in all three animals a scanty flow of gastric juice. The juice was of low acidity but rich in pepsin, and contained much mucus. The injection was followed by salivation, lachrymation, and nasal secretion; the pulse was at first slowed and then became rapid and shallow, and there was marked hyperpnea. These effects passed off within a few minutes. Gastric secretion began in about 5 minutes and continued for an hour and a half. The magnitude of the secretory effect may be illustrated by comparing it with that of the animal's regular meal on the preceding day (see table 1); it was of the same order in all three animals (8 experiments in all). Control injections of saline produced no secretion.

Since the secretion produced by choline consists largely of mucus, which does not drain readily from the pouch, and furthermore since the gastric glands at rest hold in their lumina a considerable quantity of pepsin, which may be washed out in the ferment-free fluid coming from the parietal cells, it was endeavored to obtain a clearer picture of the effect of choline on the peptic cells by superimposing its action on that of histamine, which stimulates the secretion of water and HCl but not the secretion of pepsin. Anesthetized dogs were given histamine subcutaneously (0.2 or 0.3 mgm. per kgm. of the dihydrochloride at half-hourly intervals) and choline chloride (5 to 20 mgm. per kgm.) was injected intravenously when the rate of secretion had become approximately constant. The effect of choline was uniformly (5 experiments) to raise greatly the peptic power of the juice produced by histamine, sometimes to a level approaching that of sham-feeding juice. The rate of secretion, which is very high with such doses of histamine, was generally reduced; the acidity also fell off somewhat. These results are illustrated in figure 3, which compares the effect of choline injected into *a*, the portal vein, and *b*, the jugular vein. The corresponding experiments will be discussed in detail below.

Effect of choline introduced into the digestive tract. Of more significance is the effect on gastric secretion of choline introduced into the digestive tract. This effect was conveniently studied by injecting the choline directly into the stomach through the gastric fistula. Given in this way choline, even in very large doses (0.5–1.0 gram of choline chloride in 50 cc. of water) caused no visible systemic disturbances, as it did when given intrave-

', but it did again evoke a rather scanty gastric secretion, which before was of very low acidity but rich in pepsin and mucus (table 1; see also fig. 2). The secretion began only after a latent period of about 1 hour, and lasted for about 2 hours: this supports the finding of Ivy and Javois (5) that choline acts from the intestine and not from the stomach. Equimolar solutions of NaCl (1 gram choline chloride equivalent to 0.42 gram NaCl) produced practically no secretion (fig. 2). The volume of fluid secreted, exclusive of mucus, never (11 experiments) exceeded 20 per cent of the volume secreted in response to the standard daily meal, and was usually considerably less than this.

Choline on intravenous injection, or on being introduced into the digestive tract, is therefore a weak stimulus for the secretion of fluid and HCl by the stomach, but a relatively strong stimulus for the secretion of pepsin.

TABLE 1

Gastric secretion in dogs "B" and "H" on choline and lecithin

DOG	STIMULATION	VOLUME	MUCUS	ACIDITY		PEPSIN CONCENTRATION	DURATION
				Free	Total		
		cc.	*cc.*	*m. eq./l.*	*m. eq./l.*		*hours*
B	Choline chloride (300 mgm.) intravenously	2.2	1.5	15	51	550	1¾
B	Choline chloride (1 gm.) by gastric fistula	5.3	3.6	0	20	350	2
B	Lecithin (6 gm.) by gastric fistula	4.8	3.2	0	15	230	2½
B	Daily mixed meal (average)	21.0	1.0	118	132	41	6
H	Choline chloride (300 mgm.) intravenously	3.6	1.5	0	39	460	1¾
H	Lecithin (6 gm.) by mouth	4.8	2.8	12	30	260	2
H	Daily mixed meal (average)	31.0	2.5	106	116	64	6

Effect of lecithin introduced into the digestive tract. The dose of lecithin used in all experiments was 6 grams, which contains theoretically about the same amount of choline as 1 gram of choline chloride. The choline content of the preparation was not checked. The lecithin was emulsified in 100 cc. of water at 37° and given by gastric fistula to dogs "B" and "P", or by mouth to dog "H". The latent period of the secretory effect was about 2 hours, nothing but a little mucus being discharged from the pouch in the meantime. A scanty flow of gastric juice, carrying with it considerable mucus, then began, and continued for 2 or 3 hours. As with the choline secretion, the values for free and total acid were extremely low and the values for pepsin high. The figures obtained for pepsin doubtless do not represent the entire activity of the peptic cells, since *a*, pepsin may have been destroyed by contact with alkaline mucus, and *b*, the volu clear

fluid secreted was insufficient to wash out all the pepsin discharged into the crypts of the glands; thus, when the animal was subsequently fed or injected with histamine, the first portions of the juice secreted under the new stimulus were unusually rich in pepsin.

The effect of lecithin on the gastric glands is thus qualitatively similar to that of choline. The latent period for the lecithin secretion is long, no doubt since choline is only slowly released from it by the enzymes of the intestine.

Effect of choline and of lecithin on the secretory response to a test-meal. It was noticed early in this work that, after experiments involving the administration of choline, the volume of gastric juice secreted in response to the daily mixed meal was greater than normal. This observation was extended in controlled experiments, and it was found that the secretory response to a test-meal of either lean meat or butter was augmented by previous administration of choline. The effect was not due to an overlapping of the secretion produced by the meal with the secretion produced by the "direct" effect of choline.

In the first series of experiments, dog "B" was placed on a diet consisting of 700 grams of minced lean beef muscle, given in one meal at 12:30 p.m. daily. The same lot of meat was used throughout the experimental series, which lasted 6 days. On the third day of the series, the animal received 3 hours before the meal 1 gram of choline chloride in 50 cc. of water by gastric fistula. On the fifth day it received an equivalent quantity of NaCl (0.42 gram) in the same way (fig. 1). The choline treatment augmented the secretory response to the meat meal by over 50 per cent, the augmenting effect apparently persisting to the next day. The salt control had no such effect. Two similar 6-day series of experiments gave parallel results. The pepsin concentration of the juice secreted on meat, following administration of choline, was high in the first hour only, suggesting that pepsin secreted during the direct secretory effect of choline was being washed out from the glands; thereafter the pepsin values were the low ones characterizing the juice of the Bickel pouch. Figure 2 shows the volume of secretion and the concentration of pepsin in the juice, when the stimulus was *a*, NaCl followed by meat, and *b*, choline followed by meat. The graph appears to indicate that feeding was carried out before the response to choline had ceased; however, nothing but faintly acid mucus was discharged from the pouch during the hour preceding the meal.

For subsequent experiments the animals were kept on the standard mixed diet, and experiments were not performed on successive days. The experiments were carried out in pairs, one control experiment in which the test-meal was given alone, or preceded by water or salt solution, and one experiment in which choline or lecithin was administered before the test-

[1] (The administration of water or of NaCl solution did not incre

the secretory effect of the test-meal.) Test-meals of meat or bread were given by mouth; test-meals of butter, and solutions of choline, lecithin or NaCl were always given by gastric fistula. In order to shorten the ex-

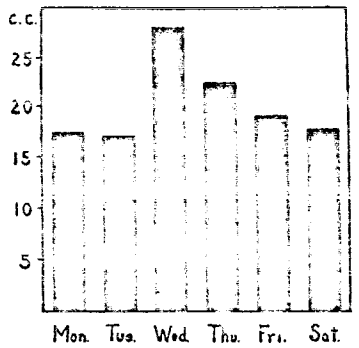

Fig. 1. Dog "B": Bickel pouch. The black rectangles represent the volume of gastric juice secreted in response to the standard daily meal of lean meat. Before the Wednesday meal the animal received 1 gram of choline chloride by gastric fistula. Before the Friday meal, it received an equivalent quantity of NaCl in the same way. Note the persistence of the augmenting effect of choline.

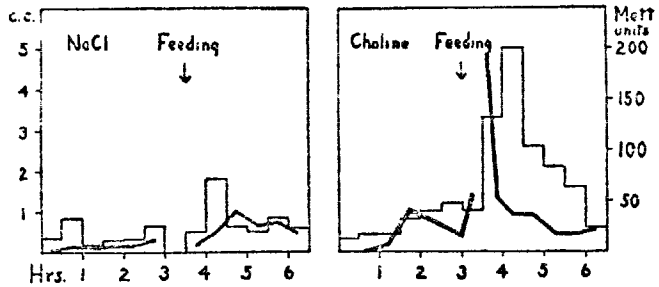

Fig. 2. Dog "B": Bickel pouch. The diagrams show the volume of juice secreted by the pouch (rectangular outlines) and the concentration of pepsin in the juice (heavy lines). The two experiments illustrated were performed two days apart under comparable conditions. In both experiments the test-meal was 700 grams of lean beef-heart, which was preceded in the first experiment by 0.42 gram of NaCl, and in the second experiment by an equivalent quantity (1 gram) of choline chloride. The secretory response to the meat meal was greatly increased by the previous administration of choline. The graph appears to indicate that feeding was carried out before the response to choline had ceased; however, nothing but faintly acid mucus was secreted during the hour preceding the meal.

periments, since the animals became very restless after the end of their normal working day, the test-meal was sometimes administered before the secretion produced by choline or lecithin had ended; since th lume of

clear juice produced by either of these substances did not, in control experiments, exceed 3 cc., this procedure introduced only a small error.

Table 2 summarizes the results of the experiments. Comparable experiments are grouped in pairs. In each set of experiments, it will be seen that the doses of choline (1 gram of choline chloride) and of lecithin (6 grams) which were used, were effective in producing a considerable increase in the volume of gastric juice secreted in response to a test-meal of meat or butter. This is still apparent when the volume of mucus is deducted from the volume of the total secretion. In the two sets of experiments in which bread was used as the test-meal this effect was practically absent. During the first two sets of experiments the dog ("B") was kept on a diet containing no added salt; hence the response to the test-meal (meat) was less than in later experiments. In one experiment (not tabulated) the secretion on meat in the same animal was lessened after lecithin: the animal appeared to be unwell at this time.

The augmentation in the secretion following the test-meal, when choline or lecithin had been administered previously, was due chiefly to an increased flow of juice in the first two or three hours after giving of the meal. The total duration of the secretory response was not affected.

The total output of HCl in all experiments closely paralleled the volume, and hence was higher when choline or lecithin had been given before the meal. The total output of pepsin (concentration x volume) was increased after choline or lecithin, but the increase, since it occurred chiefly in the first part of the test-meal secretion, was probably due, as mentioned above, to a washing-out from the glands of pepsin discharged during the primary effect of choline.

The percentage augmentation of the test-meal secretion by previous administration of choline or lecithin is given in table 3, which summarizes the results of the experiments presented in table 2. In calculating the augmentation, it was assumed that in experiments in which the test-meal was given before the secretion produced by choline or lecithin had ceased, the volume of secretion due to this was 3.0 cc. (the maximum observed in control experiments with these substances alone); and this volume was deducted from the test-meal secretion. The calculation refers to the volume of clear fluid secreted, the volume of visible mucus having been deducted. A similar augmentation by lecithin of the secretion produced by meat given by mouth was observed in the Pavlov-pouch dog (3 experiments).

The effect of choline introduced into the portal circulation. It appeared possible that the weaker secretagogue action of choline on introduction into the stomach, as compared with intravenous injection, might be due to its removal from the circulation by the liver. That the liver does exert such an effect is indicated by two experiments performed on anesthe-

TABLE 2

Gastric secretion in dogs "B," "H" and "P" on meat, butter or bread with or without previous administration of choline or lecithin

DOG	PRELIMINARY TREATMENT	TIME BEFORE TEST-MEAL	TEST-MEAL	DURATION	VOLUME		PEPSIN OUTPUT
					Total	Mucus	
		hours		*hours*	*cc.*		
B	NaCl (0.42 gm.)	3	Meat (700 gm.)	4½	4.8		160
B	Choline chloride (1 gm.)	3	Meat (700 gm.)	4½	11.3	(0.6)	805
B	Choline chloride (1 gm.)	3	Meat (700 gm.)	4	13.5	(0.1)	460
B	NaCl (0.42 gm.)	3	Meat (700 gm.)	4	9.4	(0.8)	230
B	Choline chloride (1 gm.)	3	Meat (700 gm.)	4	15.2	(0.1)	650
B	Choline chloride (0.2 gm.)*	3½	Meat (300 gm.)	5½	22.2	(2.2)	1,190
B	NaCl (0.13 gm.)*	3½	Meat (300 gm.)	5½	15.5	(1.7)	750
B	Choline chloride (1 gm.)	½	Butter (100 gm.)	10	19.6	(6.4)	1,555
B	NaCl (0.42 gm.)	½	Butter (100 gm.)	10	9.1	(7.7)	925
B	None		Butter (100 gm.)	11	13.9	(7.9)	985
B	Choline chloride (1 gm.)	½	Butter (100 gm.)	11	20.4	(7.2)	1,570
P	Choline chloride (1 gm.)	2	Butter (100 gm.)	8	16.0	(3.3)	2,860
P	None		Butter (100 gm.)	8	9.6	(1.8)	2,185
P	Choline chloride (1 gm.)	½	Butter (100 gm.)	10½	21.8	(3.2)	4,360
P	None		Butter (100 gm.)	10½	8.8	(1.5)	1,280
B	Lecithin (6 gm.)	3	Meat (500 gm.)	6	24.0	(2.5)	800
B	Water	3	Meat (500 gm.)	6	17.3	(1.5)	360
B	Lecithin (6 gm.)	3½	Meat (500 gm.)	7	21.7	(2.6)	720
B	Water	3½	Meat (500 gm.)	7	11.3	(2.4)	270
B	Lecithin (6 gm.)	½	Meat (700 gm.)	9½	39.8	(2.7)	1,900
B	None		Meat (700 gm.)	9½	29.4	(2.9)	1,100
B	Lecithin (6 gm.)	2½	Meat (500 gm.)	6	22.3	(1.8)	475
B	None		Meat (500 gm.)	6	15.7	(1.1)	355
H	Lecithin (6 gm.) + glucose (20 gm.)	4	Meat (300 gm.)	6½	30.8	(5.3)	5,030
H	Glucose (20 gm.)	4	Meat (300 gm.)	6	23.4	(3.4)	3,885
H	Lecithin (6 gm.)	½	Meat (700 gm.)	9½	41.4	(2.5)	2,920
H	Water	½	Meat (700 gm.)	9½	24.8	(1.3)	1,035
H	None		Meat (500 gm.)	7	22.0	(1.5)	1,145
H	Lecithin (6 gm.)	3½	Meat (500 gm.)	7	28.1	(1.5)	2,845

* Injected intravenously.

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TABLE 2—*Concluded*

DOG	PRELIMINARY TREATMENT	TIME BEFORE TEST-MEAL	TEST-MEAL	DURATION	VOLUME		PEPSIN OUTPUT
					Total	Mucus	
		hours		*hours*	*cc.*		
B	Lecithin (6 gm.)	½	Butter (100 gm.)	9½	15.9	(8.1)	1,605
	NaCl (0.42 gm.)	½	Butter (100 gm.)	9½	13.9	(8.8)	750
B	Lecithin (6 gm.)	½	Butter (100 gm.)	8½	12.2	(3.6)	650
	None		Butter (100 gm.)	7½	6.9	(5.0)	405
H	Lecithin (6 gm.)	½	Butter (100 gm.)	8	11.1	(3.5)	1,040
	None	½	Butter (100 gm.)	8	4.4	(3.5)	245
P	None	½	Butter (100 gm.)	10	8.8	(1.5)	1,280
	Lecithin (6 gm.)	½	Butter (100 gm.)	10	18.8	(2.9)	3,535
B	None	½	Bread (250 gm.)	10	10.4	(3.9)	795
	Lecithin (6 gm.)	½	Bread (250 gm.)	10	13.8	(6.0)	920
H	None	½	Bread (250 gm.)	8	16.3	(6.5)	2,025
	Lecithin (6 gm.)	½	Bread (250 gm.)	8	22.0	(8.2)	3,795

TABLE 3

NUMBER OF PAIRS OF EXPERIMENTS	TEST-MEAL	PRELIMINARY TREATMENT	AUGMENTATION	
			Range	Average
			per cent	*per cent*
8	Meat	Choline chloride (1 gm.)	44-108	84
7	Meat	Lecithin (6 gm.)	25-116	44
4	Butter	Choline chloride (1 gm.)	50-320	130
4	Butter	Lecithin (6 gm.)	0-400	140
2	Bread	Lecithin (6 gm.)	−10- 10	0

tized dogs secreting under the influence of histamine. In these experiments, in a dog secreting in response to histamine, the same dose of choline chloride was injected slowly into *a*, a branch of the superior mesenteric vein, and *b*, the jugular vein, the technique for collection of juice being as described under "Methods". The increase in the output of pepsin following injection *a* was less than half of that produced by injection *b*, and did not last so long. Reversing the order of the injections did not change the results. Figure 3 represents the results of one of the experiments.

The effect of atropine on the secretory action of choline. The direct secretory action on the gastric glands of choline, given either intravenously or by gastric fistula, is fully antagonized by atropine (0.2 mgm. atropine sulfate per kgm.). Whether or not the secondary augmenting action of

choline is also antagonized could not be ascertained, since atropine interfered with the digestion of the test-meal.

DISCUSSION. The experiments show definitely that besides its direct secretory effect on the gastric glands, choline is capable of augmenting their response to a meal of meat or butter. Lecithin can act similarly. The way in which choline exerts this "augmenting" action on the secretion is not easily understood. It is possible that an increased secretion of pepsin (and possibly of the pancreatic enzymes as well) under the "direct" action of choline, might hasten the escape of secretagogues from the digesting food, and so cause an increased stimulation of the gastric glands. Against this supposition is the fact that the duration of the test-meal secretion is not shortened by choline. It seems more probable that there

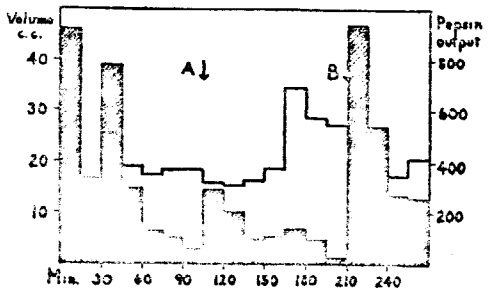

Fig. 3. The heavy rectangular line represents the volume of gastric juice secreted in each 15-minute period by an anesthetized dog receiving 0.2 mgm. of histamine dihydrochloride per kgm. subcutaneously every 30 minutes. The shaded areas represent the total output of pepsin during each period. Note the steady decline in pepsin secretion after the first 45 minutes. At *A*, 100 mgm. of choline chloride were injected into a branch of the superior mesenteric vein; at *B*, the same dose was injected into the jugular vein. Note the greater effect on enzyme discharge of *B* as compared with *A*. Choline had no striking effect on the volume of the secretion produced by histamine.

is some sort of sensitization of the parietal cells themselves, but how this might take place there is nothing to indicate. It is worthy of note that the sensitization affects chiefly the parietal cells, which secrete the fluid and the hydrochloric acid of the juice, and not the peptic cells. The secretory response of the stomach to a standard stimulus may undoubtedly be modified by the state of nutrition of the experimental animal. Thus Koschtoyantz (8) has shown that the prolonged administration of a meat diet increases the response of a denervated pouch to various test-meals; and the addition of liberal quantities of salt to the diet is well known to have a similar effect.

Lecithin is hydrolyzed by the digestive ferments (7), and it is therefore not surprising that its effect should be qualitatively similar to that of

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choline. Whether or not choline in the form of lecithin is as effective a stimulus as the free base, cannot be concluded from these experiments, in view of the wide variations between the augmentations observed in the individual experiments. The efficiency of choline and lecithin, when comparable quantities of each are used, appears, however, to be of the same order. The literature does not indicate whether the choline of ingested lecithin reaches the blood as such, or whether it is used to resynthesize the phosphatide in the intestinal wall.

Choline has a stronger secretagogue action when given intravenously than when introduced into the digestive tract. The experiments on intraportal injection of choline suggest that this may be due to its being taken up in the latter case by the liver.

Since the choline of lecithin, as well as any free choline of the diet, can act on the gastric glands, it is of interest to know whether the base is present in the diet in sufficient quantity to be an important factor in the production of the second phase. The doses of choline used in these experiments are certainly much higher than the best figures in the literature for *free* choline in, *e.g.*, skeletal muscle: the true values for the latter are probably very low (9). Recent work has shown, however, that choline is readily liberated by post-mortem autolysis from a water-soluble precursor present in many tissues (9, 10), so that very large quantities of choline have been found in tissues which were not fixed by heating immediately on removal from the body. Thus liver has been found to contain up to 720 mgm. per kgm. (11), and skeletal muscle 150 to 200 mgm. per kgm. (12, 13), even when promptly worked up; this liberated choline is not destroyed by further autolysis. Commercial meats may therefore be presumed to contain very considerable quantities of free choline. The *total* choline of a large number of foods has been determined by Fletcher, Best and Solandt (14). Among other values, liver (dog) was found to contain 2300 mgm. per kgm., beef muscle 750 mgm. per kgm., and white wheat flour 1400 mgm. per kgm. Thus a meat meal of 700 grams, such as was fed in these experiments, contains about 500 mgm. of choline, or about three-fifths the quantity (1 gram of choline chloride) which was found to produce an augmentation averaging 84 per cent in the secretory response to such a meal. The secretion produced by the meat in control experiments was thus probably due, in considerable part, to the "augmenting" effect of the choline which it contained.

In contrast, the "direct" action on the parietal cells of choline derived from food cannot be of much importance in the production of the second phase. Thus in table 1, a dose of choline considerably greater than that which would occur in the daily meal, produced less than one-tenth the volume of (clear) gastric juice produced by the meal itself. On the other hand, the output of pepsin following this dose of choline was twice as

great as that following the meal. Table 2 shows that both lecithin and free choline, in the doses used, markedly stimulate the discharge of pepsin. Since this dose does not greatly exceed the quantity of choline available in a high-protein diet, it is practically certain that the choline of the diet may be a factor in promoting the discharge of pepsin, and that the second phase may involve the activity of the peptic cells. This is evident from the fact that the gastric juice secreted in response to histamine, which stimulates almost exclusively the parietal cells, contains far less pepsin than the gastric juice of the second phase. In the normally innervated stomach, in which the peptic cells undergo intense stimulation through the vagi, this effect of choline would of course be less important.

It is of interest to note that the secretagogue action of choline could be regularly observed in a completely denervated stomach pouch, the test being made at frequent intervals six weeks to fifteen months after denervation. This finding may be contrasted with the statement of Suda (15) that acetylcholine loses its secretory effect on complete denervation of the pouch.

SUMMARY

1. Choline administered to dogs intravenously or by gastric fistula is a weak stimulus for the secretion of fluid and acid by the gastric glands, but a comparatively strong stimulus for the secretion of pepsin.

2. The secretagogue action of lecithin is similar to that of choline, allowance being made for the delay in liberation of choline from lecithin in the gut.

3. The volume of gastric juice secreted in response to test-meals of meat or butter is markedly augmented by previous administration of choline or lecithin. This "augmenting" effect of choline and lecithin is a secondary one, and persists after the direct secretory effect has passed off.

4. The quantities of choline available in the diet indicate that the direct secretory action of choline is not an important factor in the production of the second phase of gastric secretion. The secondary augmenting action of choline, however, plays an important, but subsidiary, part in the production of the second phase.

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Phospholipids of rat tissues after feeding pure phosphatidyl ethanolamine and lecithin

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ABSTRACT Pure phosphatidyl ethanolamine and lecithin from egg yolks were fed to rats in saline or in olive oil and the changes in individual phospholipids in the intestinal wall, liver, and plasma of the animals were studied.

Ingestion of olive oil alone produced increased levels of all phospholipid fractions in each of the three tissues. Feeding phosphatidyl ethanolamine in saline resulted in slightly increased plasma phospholipids, but levels of liver total phospholipids were greatly reduced; when phosphatidyl ethanolamine was fed with olive oil, liver phospholipids were again reduced but this reduction was confined to the phosphatidyl ethanolamine and phosphatidic acid fractions. Feeding lecithin alone did not produce significant changes in levels of plasma or tissue phospholipids.

The results suggest that liver phospholipid synthesis is depressed by feeding phosphatidyl ethanolamine; in the presence of olive oil, hepatic synthesis of phosphatidyl ethanolamine seems to be more selectively inhibited.

KEY WORDS phospholipids · intestinal wall · liver · plasma · force-feeding · olive oil · lecithin · phosphatidyl ethanolamine · inhibition · hepatic synthesis · rats · egg yolk · gradient elution

ISOTOPIC STUDIES on the synthesis of total phospholipids by various animal tissues in vitro have been reported extensively (1, 2) and similar studies in the living animal have been carried out by Artom and Swanson (3, 4). However, less is known of the absorption of purified single phospholipids, with which the present paper is concerned. After we had established that fed phospholipids were absorbed from the intestinal lumen of the rat to the extent of 96% within 4 hr, we studied the effects of feeding purified phosphatidyl ethanolamine (PE) and lecithin on the levels of individual phospholipids of the rat intestinal wall, plasma, and liver after this time interval. Since the phospholipids were fed in saline or in olive oil, the effect of the oil alone on phospholipid synthesis was also studied.

In a previous study, Hill, Linazasoro, Chevallier, and Chaikoff (5) (who were concerned mainly with glucose metabolism in relation to fat feeding) observed that feeding certain fats to rats over a period of 3 days with 50% glucose in the diet did not affect the lipid levels of the liver or the phospholipid levels of plasma. It appears from the present work that feeding fat alone does influence the levels of phospholipids in the liver and the plasma. The changes in levels of plasma and liver phospholipids produced during a single feeding are obviously due to a combination of endogenous changes and absorption from the intestine. The effects of absorption have been further studied by feeding radioactively labeled phospholipids. These results will be reported later.

MATERIALS AND METHODS

Animals

Male Wistar albino rats, 8–12 wk of age and weighing 150–200 g, were used. Before feeding, the animals were randomly distributed amongst the cages and left overnight with water but without food. Materials were administered by stomach intubation using rubber catheters (English gauge 3). The animals were lightly anesthetized with ether so that they were fully recovered by the time the catheter was removed (about 30 sec). The total volume fed was usually 1.5 ml and this was followed by 0.5 ml of saline.

Preparation of Food

Phospholipid (250 mg) or olive oil (400 mg) or 250 mg

Abbreviations: PA, phosphatidic acid; PE, phosphatidyl ethanolamine; LPE, lysophosphatidyl ethanolamine; TLC, thin-layer chromatography.

phospholipid + 400 mg of olive oil were mixed with egg white (0.15 ml) and saline to give a total volume of 1.5 ml. The mixture was emulsified in an ultrasonic vibrator with external cooling (0°C) until it was homogeneous (2–5 min). Sufficient food for 8–10 animals was prepared in the above proportions and 1.5 ml of emulsion was fed from a syringe to each animal as described above. The amount of lipid administered was calculated from the organic phosphorus content of the food in the case of phospholipids and from the total ester content in the case of olive oil, after subtracting the amount of residual lipid in the catheter and feeding vessel.

Preparation of Pure PE and Lecithin from Eggs

Egg yolks were stirred with a little water and a volume (x ml) of the mixture poured into a mechanically stirred mixture of chloroform ($3x$ ml) and ethanol ($6x$ ml). The mixture was heated under reflux (in an atmosphere of nitrogen) for 15 min. The precipitated proteins were filtered under suction through Whatman No. 1 paper and the filtrate was evaporated under reduced pressure to a low volume. Acetone (20 vol) was added and the mixture allowed to stand at 4°C overnight. The precipitated crude phospholipids were collected by filtration. They were redissolved in $CHCl_3$-CH_3OH 1:1 (v/v) 50 ml, and the extract was washed with 0.1 N HCl (3 vol). After standing for some hours the $CHCl_3$ layer was separated and the solvent was evaporated in vacuo.

The residual crude phospholipids (which still contained a substantial amount of triglycerides) were dissolved in dry chloroform and further purified by chromatography.

Silicic acid (Mallinckrodt Chemical Works, New York) 100 mesh was treated as described by Billimoria, Curtis, and Maclagan (6). A portion of the dry $CHCl_3$ extract containing phospholipid (not exceeding 5 g) was applied to a column (5.6 cm diam) containing 150 g of silicic acid and the column was eluted with dry $CHCl_3$ (3 liters) to remove all triglycerides and free and esterified cholesterol. The column was transferred to a gradient elution apparatus (7) and the individual phospholipids were separated by elution with a linearly increasing concentration of CH_3OH in $CHCl_3$. The separation was monitored by phosphorus estimations and the separated peaks of PE, lysophosphatidyl ethanolamine (LPE), lecithin, and sphingomyelin were individually pooled. PE and lecithin fractions were analyzed as described under chemical estimations.

Rat Tissue Phospholipids

Fed animals were anesthetized with ether 4 hr after feeding. Blood was collected from the heart and after exsanguination the animals were killed. The liver, intestine, and stomach were removed and the intestinal walls were slit and washed thoroughly with saline. Stomach washings were similarly obtained. Phospholipids and esters in the washings were estimated after extraction with Bloor's solvent (ethanol-ether 3:1) in order to determine the amount of lipid not absorbed.

The intestine and liver were separately ground with pestle and mortar under Bloor's solvent and then thoroughly extracted with several further portions of the boiling solvent. The extracts were filtered and made up to a known volume. An aliquot was removed for estimation of organic phosphorus. The solvent was removed from the residual extract and the crude lipids were dissolved in dry $CHCl_3$, filtered under centrifugal force through a Seitz filter, and chromatographed.

Blood was centrifuged for 10 min at 4°C at 3,500 × g and the plasma separated. The latter (1 vol) was extracted with Bloor's solvent and made up to 20 vol with the solvent. An aliquot was reserved for phosphorus estimation and the rest of the extract was chromatographed.

Usually phospholipids of liver or intestine from the organs of individual animals were fractionated, but plasma (8–10 ml) was pooled from at least two animals in order to obtain sufficient material.

Identification of Phospholipids

The purified phospholipid fractions were identified by TLC. Organic phosphorus was estimated by a modification of the method of Allen (8), as described previously (6). Values of phospholipids were obtained by multiplying the phosphorus content by 25. Amino nitrogen was estimated by the method of Lea and Rhodes (9), except that the ninhydrin reagent was prepared according to Jacobs (10). Total nitrogen was estimated after digestion of the lipid with perchloric acid (11), using the Nessler reagent according to King and Wootton (12); NaI was substituted for KI in the Nessler reagent to avoid formation of the insoluble potassium perchlorate. Choline was determined according to Böttcher, Pries, and Van Gent (13). Bases were identified by amino acid chromatography after hydrolysis of the lipids with 6 N HCl at 100°C in sealed tubes for 6 hr (14). The inositol content of each fraction was determined by the microbiological assay of Norris and Darbre (15) and glycerophosphoryl bases were identified by the paper chromatographic technique of Dawson (16). Ester estimations were carried out according to Rapport and Alonzo (17). The fatty acid compositions of the lipids fed were determined by gas-liquid chromatography on ethylene glycol adipate polyester (15% on Celite, 100–120 mesh) at 174°C and a flow rate of 44 ml/min using an argon ionization detector as previously described by Billimoria, Irani, and Maclagan (18).

RESULTS

Purity of Lipids Fed

The PE fraction gave a single spot on TLC and behaved identically with a sample of synthetic PE prepared by Billimoria and Lewis (19). Its nitrogen was all present as amino nitrogen and the P/N ratio was 1:1.01. A trace of serine was detected by paper chromatography after hydrolysis. The ester/P ratio (1.91–1.0) was a little lower than the theoretical value of 2.0:1. Some (1–3%) of the PE was present in the plasmalogen form. LPE was completely absent as indicated by a negative red cell lysis test and by TLC.

Lecithin was free from sphingomyelin and gave a P/N ratio of 1:1.01, showed complete absence of amino nitrogen, and gave a P/ester ratio of 1:2.0.

TABLE 1 FATTY ACID COMPOSITION OF EGG YOLK PHOSPHOLIPIDS

	PE	Lecithin
	% of total fatty acid	
Myristic	tr.	tr.
Palmitic	17.8	43.0
Palmitoleic	—	tr.
Stearic	33.4	9.5
Oleic	25.7	27.0
Linoleic	9.5	16.0
Arachidonic	10.7	1.7
Behenic	2.9	3.0

Tissue Phospholipid Fractions

The fraction shown in Fig. 1 as phosphatidic acid contained only a trace of nitrogen as impurity and on TLC ran close to the solvent front, its R_f being identical with a synthetic marker of β,γ-dipalmitoyl L-α-phosphatidic acid prepared by the method of Hessel, Morton, Todd, and Verkade (20). After mild alkali hydrolysis, chromatography (16) established the presence of glycerophosphoric acid. The PE fractions from plasma, liver, and intestine were pure but the lecithin fractions contained some sphingomyelin. Further separation of the lecithin from sphingomyelin was unnecessary as lecithin and sphingomyelin phosphorus could be separately estimated from the mixture after mild alkali hydrolysis by the method of Dawson (21). The LPE fraction, although giving on TLC only one spot corresponding to the required lipid, contained traces of adhering amino acids in spite of the washing procedure described in the Methods

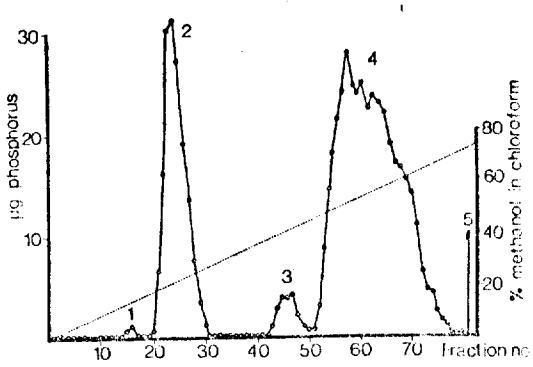

FIG. 1. Separation of egg yolk phospholipids by continuous linear gradient elution chromatography. Crude phospholipids (4.15 g) were applied to silicic acid (150 g). Column was eluted with $CHCl_3$–CH_3OH with increasing concentration of CH_3OH (right-hand ordinate). Fractions of 25 ml were collected. Micrograms of P from an aliquot (0.1 ml) are plotted against fraction number. Broken line, gradient of CH_3OH–$CHCl_3$. Peaks: *1*, phosphatidic acids; *2*, PE; *3*, LPE containing 20% w/w phosphatidyl inositol; *4*, lecithins; *5*, sphingomyelins and lysolecithins (250 mg), eluted with C_2H_5OH–$CHCl_3$–H_2O 6:3:1 (v/v).

TABLE 2 INTESTINAL WALL PHOSPHOLIPIDS 4 HR AFTER FEEDING LIPIDS

Results expressed as mg/100 g tissue ± SEM with statistical significance of difference from control values in parentheses

	Lipids Fed					
	None	Olive Oil	PE/Saline	Lecithin/Saline	PE/Olive Oil	Lecithin/Olive Oil
No. of expts. (animals)	6 (6)	6 (6)	7 (7)	8 (8)	8 (8)	6 (6)
PA	19.5 ± 5.7	9.8 ± 1.2 (NS)	31.6 ± 3.3 (NS)	25.1 ± 2.1 (NS)	58.8 ± 10.0 ($P < 0.01$)	28.8 ± 3.5 (NS)
PE	159.3 ± 26.2	210.8 ± 18.7 (NS)	109.4 ± 10.2 (NS)	170.1 ± 11.5 (NS)	113.5 ± 10.8 (NS)	155.0 ± 9.3 (NS)
LPE	45.1 ± 1.6	58.6 ± 4.9 ($P < 0.05$)	44.4 ± 4.8 (NS)	41.5 ± 4.2 (NS)	59.0 ± 3.1 ($P < 0.01$)	46.4 ± 3.8 (NS)
Total cephalin (PE + LPE)	204.4 ± 28.6	269.4 ± 21.5 (NS)	153.8 ± 8.3 (NS)	211.6 ± 11.5 (NS)	172.5 ± 11.6 (NS)	201.4 ± 9.3 (NS)
Lecithin	281.0 ± 43.7	392.8 ± 18.6 ($P < 0.05$)	205.5 ± 19.1 (NS)	374.3 ± 42.6 (NS)	251.3 ± 20.3 (NS)	326.2 ± 19.4 (NS)
Sphingomyelin	44.4 ± 3.5	63.3 ± 2.3 ($P < 0.001$)	43.5 ± 3.1 (NS)	64.2 ± 9.6 (NS)	45.4 ± 3.3 (NS)	61.4 ± 5.1 ($P < 0.05$)
Total phospholipids	549.3 ± 80.6	735.2 ± 39.9 ($P = 0.05$)	434.3 ± 24.3 (NS)	675.2 ± 47.2 (NS)	528.0 ± 31.9 (NS)	617.9 ± 33.2 (NS)

TABLE 3 PLASMA PHOSPHOLIPIDS 4 HR AFTER FEEDING LIPIDS
Results expressed as mg/100 ml plasma ± SEM with statistical significance in parentheses

	Lipids Fed					
	None	Olive Oil	PE/Saline	Lecithin/Saline	PE/Olive Oil	Lecithin/Olive Oil
No. of expts. (animals)	4 (53)	4 (48)	4 (8)	4 (8)	4 (8)	4 (8)
PA	1.0 ± 0.3	1.2 ± 0.1 (NS)	5.0 ± 1.7 (NS)	0.9 ± 0.1 (NS)	4.4 ± 1.2 ($P < 0.05$)	1.3 ± 0.2 (NS)
PE	4.6 ± 1.4	8.1 ± 1.2 (NS)	11.7 ± 3.5 (NS)	2.8 ± 0.8 (NS)	11.4 ± 0.8 ($P < 0.01$)	3.6 ± 0.6 (NS)
LPE	2.9 ± 0.9	7.1 ± 1.1 ($P < 0.05$)	7.9 ± 2.7 (NS)	4.0 ± 0.9 (NS)	3.5 ± 1.1 (NS)	2.3 ± 0.5 (NS)
Total cephalin (PE + LPE)	7.5 ± 0.5	15.1 ± 2.3 ($P < 0.02$)	19.0 ± 3.2 ($P < 0.01$)	6.8 ± 1.5 (NS)	14.9 ± 1.0 ($P < 0.001$)	5.9 ± 0.8 (NS)
Lecithin	83.2 ± 3.1	110.1 ± 3.7 ($P < 0.01$)	120.0 ± 13.9 (NS)	82.6 ± 7.3 (NS)	106.7 ± 5.7 ($P < 0.02$)	98.5 ± 1.3 ($P < 0.01$)
Sphingomyelin	7.9 ± 0.7	11.7 ± 0.2 ($P < 0.01$)	11.4 ± 1.3 ($P < 0.05$)	9.1 ± 0.2 (NS)	12.1 ± 1.7 (NS)	9.2 ± 1.2 (NS)
Total phospholipids	99.6 ± 1.9	138.1 ± 3.5 ($P < 0.001$)	137.9 ± 15.5 ($P < 0.05$)	99.4 ± 5.3 (NS)	137.9 ± 6.9 ($P < 0.01$)	115.0 ± 2.6 ($P < 0.01$)

TABLE 4 LIVER PHOSPHOLIPIDS 4 HR AFTER FEEDING LIPIDS
Results expressed as mg/100 g tissue ± SEM with statistical significance in parentheses

	Lipids Fed					
	None	Olive Oil	PE/Saline	Lecithin/Saline	PE/Olive Oil	Lecithin/Olive Oil
No. of expts. (animals)	6 (12)	6 (12)	9 (9)	8 (8)	8 (8)	8 (8)
PA	147.4 ± 40.8	284.3 ± 23.7 ($P < 0.02$)	62.9 ± 12.8 (NS)	137.1 ± 18.4 (NS)	89.1 ± 11.0 (NS)	123.4 ± 11.7 (NS)
PE	934.8 ± 103.2	1225.8 ± 19.4 ($P < 0.02$)	595.9 ± 28.8 ($P < 0.01$)	942.9 ± 64.8 (NS)	469.5 ± 71.7 ($P < 0.01$)	920.0 ± 30.6 (NS)
LPE	77.0 ± 9.9	59.0 ± 2.8 (NS)	61.7 ± 5.9 (NS)	120.3 ± 10.8 ($P < 0.02$)	97.6 ± 11.4 (NS)	110.9 ± 12.1 ($P < 0.05$)
Total cephalin (PE + LPE)	1011.8 ± 99.1	1284.7 ± 21.3 ($P < 0.05$)	657.6 ± 33.3 ($P < 0.01$)	1063.2 ± 70.3 (NS)	567.1 ± 81.6 ($P < 0.01$)	1030.9 ± 107.8 (NS)
Lecithin	1403.0 ± 81.5	1814.9 ± 45.8 ($P < 0.001$)	1076.6 ± 111.5 ($P < 0.05$)	1610.3 ± 77.9 (NS)	1398.9 ± 51.9 (NS)	1628.4 ± 165.1 (NS)
Sphingomyelin	93.0 ± 5.5	124.6 ± 5.1 ($P < 0.01$)	105.8 ± 14.1 (NS)	119.3 ± 10.2 ($P < 0.05$)	79.1 ± 11.4 (NS)	124.9 ± 14.5 (NS)
Total phospholipids	2655.3 ± 129.0	3508.5 ± 38.2 ($P < 0.001$)	1901.2 ± 85.1 ($P < 0.001$)	2930.1 ± 151.6 (NS)	2134.1 ± 86.9 ($P < 0.01$)	2907.6 ± 260.4 (NS)

section. This fraction also contained almost all the inositol. Over 80% of this fraction was present as LPE, the remaining 20% being phosphatidyl inositol. The red cell lysis reaction was strongly positive.

A separation of the egg phospholipids on silicic acid columns by linear gradient elution is shown in Fig. 1. Only the PE and lecithin peaks were used in the feeding experiments.

The fatty acid compositions of the PE and lecithin fed are shown in Table 1. Of the saturated acids PE contained mainly stearic acid whereas lecithin contained mainly palmitic acid. Of the unsaturated acids the oleic acid content was similar in both lipids but PE contained more arachidonic acid and lecithin more linoleic acid.

Levels of phospholipid fractions in the intestinal wall, plasma, and liver after feeding olive oil and (or) phospholipids are shown in Tables 2, 3, and 4 respectively. In the plasma of unfed animals the major phospholipid is lecithin (83%) and only 8% of cephalin is present. In the liver and intestine higher levels of cephalin (38%) are found. Relatively low levels of LPE (3%) are found in plasma and liver but as much as 8% is present in intestine. The reported phospholipase A activity (22) in intestinal mucosa may account for the higher LPE content of the organ. It is unlikely that this is a cleavage product of either PE or plasmalogen arising as an artifact of chromatography since, under similar conditions, synthetic PE and the small amounts of plasmalogen PE from egg yolk are recovered intact.

The effects of feeding PE differed significantly from those of feeding lecithin, and these differences are summarized in Table 5. The major phospholipid fractions of

TABLE 5 DIFFERENCES IN TISSUE PHOSPHOLIPIDS AFTER FEEDING PE AND LECITHIN

Results given as the difference (amount when lecithin fed) − (amount when PE fed) ± SEM. The statistical significance is shown in parentheses

	Intestine	Plasma	Liver
	mg/100 g wet wt	*mg/100 ml*	*mg/100 g wet wt*
PA	−10.1 ± 3.9 ($P < 0.05$)	−4.1 ± 1.7 ($P = 0.05$)	+74.2 ± 22.4 ($P < 0.01$)
PE	+60.7 ± 15.3 ($P < 0.01$)	−8.9 ± 3.6 ($P = 0.05$)	+347.0 ± 70.9 ($P < 0.001$)
LPE	−2.9 ± 6.3 (NS)	−3.9 ± 2.8 (NS)	+58.6 ± 12.2 ($P < 0.001$)
Total cephalin	+57.8 ± 14.1 ($P < 0.01$)	−12.8 ± 3.5 ($P < 0.02$)	+405.6 ± 77.7 ($P < 0.001$)
Lecithin	+168.8 ± 46.7 ($P < 0.01$)	−19.4 ± 15.7 (NS)	+533.7 ± 136.0 ($P < 0.01$)
Sphingomyelin	+20.7 ± 10.1 (NS)	−2.3 ± 1.3 (NS)	+13.5 ± 17.4 (NS)
Total phospholipids	+240.9 ± 53.1 ($P < 0.001$)	−40.8 ± 16.4 ($P = 0.05$)	+1028.9 ± 174.1 ($P < 0.001$)

liver and intestine were increased and those of plasma decreased when PE was replaced by lecithin in the feeding experiments.

DISCUSSION

Our results indicate that a single meal of olive oil gives rise to large changes in phospholipid levels of plasma, intestinal wall, and the liver of the rat, which were raised by as much as 30-40% in each of the tissues studied. These increases were particularly prominent in the liver and were distributed over almost all the phospholipid fractions.

In fat-feeding experiments Hill et al. (5) have shown that the feeding of fat-containing diets (corn oil and vegetable oils) over a period of 3 days did not influence the lipid content of the liver or the lipid, including phospholipid, levels of the plasma. The striking difference in our results may be due to differences in dietary composition. Whereas the diet of Hill et al. contained as much as 50% glucose we fed fat alone. The difference in the two results is of sufficient interest to warrant further investigation as it is conceivable that administration of fat alone may result in a fatty liver, which may be avoided by the addition of carbohydrate to the feed.

The two pure lipids, PE and lecithin, have totally different effects on lipid levels of plasma, intestine, and liver when fed individually.

On feeding PE the levels of total phospholipids in the liver were strikingly reduced; this reduction was observed both in the lecithin and PE fractions. A similar trend was observed in the intestine, although the changes here were not significant. The larger scatter of the results is probably due to absorption from the intestine still occurring 4 hr after feeding. The plasma total phospholipids were raised after the feeding but the plasma PE level was not significantly increased, the increase being uniformly distributed over the other phospholipid fractions.

Two possible explanations of the reduced content of liver phospholipids after feeding PE are (*a*) inhibition of liver synthesis of phospholipids or (*b*) increase in the rate of destruction of phospholipids. The latter suggestion seems less likely, as an increased destruction would be associated with increased levels of the intermediate metabolite PA which were not observed; in fact, PA levels were reduced from 147 to 63 mg/100 g wet weight, suggesting decreased breakdown. When PE was fed with olive oil, the liver PE was decreased by over 60%, whereas the lecithin levels were almost unchanged. It is therefore suggested that the reduced levels of phospholipids represent an inhibition of synthesis. The subject is still under investigation.

Similar results were not observed when lecithin was fed; instead the lecithin contents of intestine, plasma, and liver were all slightly raised. The fact that plasma and liver lecithin levels are not significantly raised after feeding lecithin suggests that its turnover must be very rapid, in agreement with Gurr, Pover, Hawthorne, and Frazer (23).

Our interest in the experiments was concerned partly with the well known effects of fat feeding on blood coagulation, which appear to depend mainly on the PE fraction (24). Our results indicate that feeding PE and (or) olive oil causes large increases in the plasma PE fraction, which would explain the accelerated coagulation. Feeding lecithin with olive oil, however, abolishes the rise in plasma PE; lecithin might therefore merit trial as a desirable adjuvant to dietary fats.

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THE EFFICIENCY OF UTILIZATION OF PHOSPHORUS BY THE ALBINO RAT[1]

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The purpose of this study was to compare the rates of absorption and utilization of phosphorus as present in phytin, in lecithin and in disodium phosphate; especially in relation to the pH of the alimentary contents and the phytin-splitting enzyme of the intestinal wall, and as the facts in these matters contribute to the understanding of the rachitogenic property of cereals.

Kinsman et al. ('39) gave a method for expressing the utilization of calcium in the following terms:

$$\frac{\text{Retention, Period A} - \text{Retention, Period B}}{\text{Intake, Period A} - \text{Intake, Period B}} \times 100 = \text{per cent utilization}$$

This method of representing efficiency of utilization was followed in the present investigation, the phosphorus intake in period A being high but not in excess of the level of maximum efficiency of utilization; and in period B the intake of phosphorus was just above the level of equilibrium.

The rachitogenic property of cereals was attributed by Bruce and Callow ('34) to the unavailability of the phytin phosphorus present. Harrison and Mellanby ('39) found that commercial phytin was not rachitogenic, whereas, sodium phytate was rachitogenic. They suggested that the rachitogenic action of cereals is normally due to the action of the phytic acid in inhibiting the absorption of calcium. Palmer and Mottram ('39) indicated that the unavailability of phytin phosphorus is associated with the disproportion of the levels of calcium and phosphorus fed.

Lowe and Steenbock ('36b) found that phytin phosphorus was absorbed although not as efficiently as inorganic phosphorus; but when the ration contained 3% calcium carbonate scarcely any of the phytin phosphorus was absorbed. They postulated that calcium carbonate

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inhibits the action of an intestinal enzyme which normally hydrolyzes phytin and makes its phosphorus available. In further studies Krieger et al. ('40a) comparing inositol-hexaphosphoric acid with inorganic phosphorus at a Ca/P ratio of 1:1, found the phytin phosphorus almost as readily utilized as the inorganic phosphorus. However, when the Ca/P ratio was 2:1 the availability of the phytin phosphorus was markedly decreased, while that of the inorganic phosphorus was not markedly affected. Jones ('39) also found that for rats the Ca/P ratio had a marked effect on the availability of phytin phosphorus. Krieger et al. ('40b) found the calcium of calcium phytate to be as available as that of calcium carbonate. McCance and Widdowson ('35) have shown that of the total phosphorus of cereals between 41 and 68% is phytin phosphorus, and that from 20 to 60% of the phytin fed to humans is excreted unchanged in the feces.

Eastman and Miller ('35), reporting on intestinal pH values, found the pH higher in rats fed a rachitogenic diet than in rats fed a normal diet.

Patwardhan ('37) found, in extracts from the intestinal wall of rats, an enzyme capable of the hydrolysis of sodium phytate. Patwardhan ruled out the possibility of the enzyme coming from the food because the enzyme was found present in weanling rats. Lowe and Steenbock ('36b) were of the opinion that any hydrolysis of the phytin which takes place in the intestines is due to the activity of the intestinal flora or to the phytase from the vegetable part of the diet.

EXPERIMENTAL

The experiment was carried out using growing albino rats as subjects. These animals were fed a commercial dog food[2] ad libitum until they reached a weight of about 80 to 90 gm. From this time on they received a basal ration in which phosphorus was present to the amount of 0.12%. This approximates the minimum amount for some growth. Following this basal period the rats were fed rations with double this amount of phosphorus, 0.25%. The extra phosphorus was in the form of disodium phosphate, of crude soy bean lecithin,[3] or of phytin.[4] In all cases the Ca/P ratio was approximately 2:1. The 0.12% phosphorus chosen for the basal ration was below the amount indicated by Forbes ('37) as being near the minimum, and not lower than the 0.113% indicated by Brown et al. ('32) as producing rickets.

[2] Purina dog chow.
[3] A. E. Staley Mfg. Co.
[4] Pfanstiehl Chemical Co.

For the higher phosphorus rations, 0.25% was chosen as being below the 0.257% indicated by Nicolaysen ('37) as that required for optimum growth.

The basal rations, per 1,000 gm., contained 60.0 gm. of wheat gluten, 120.0 gm. of lactalbumin, 538.1 gm. of corn sugar,[5] 80.0 gm. of a hydrogenated fat,[6] 70 gm. of butter, 26.0 gm. of calcium and phosphorus-free salt mixture (Sherman and Smith, '31), 30.0 gm. of cod liver oil, 64.0 gm. of milk vitamin concentrate,[7] 5 mg. of thiamine hydrochloride, 2.45 gm. of disodium phosphate, 2.68 gm. of calcium carbonate, and 6.77 gm. of sodium bicarbonate. The higher phosphorus rations had essentially the same composition except that the sodium bicarbonate was omitted and additional phosphorus was added as needed in the form of disodium phosphate, crude soy bean lecithin, or phytin. Since the crude lecithin contained some oil the amount of fat was cut down to 41.8 gm. for this ration. Calcium carbonate was added as needed to bring the C/P ratio to 2:1. In all cases the adjustment to 1,000 gm. of ration was made at the expense of the corn sugar. All rats were fed 7.14 gm. of ration per day.

Rats 1 to 36 used in basal period I and in period II were litter-mate triplets (table 1). In period II, on the higher phosphorus, one rat of the triplet received the disodium phosphate ration, a second the lecithin ration, and the third the phytin ration. Periods I and II were 14 days long with collection of urine and feces during the last 7 days in each case. Rats 37 to 64 used in basal period III and in period IV, were litter-mate pairs. In period IV, on the higher phosphorus, the first rat of the pair received the disodium phosphate ration and the second the phytin ration. Periods III and IV were 21 days long with collection of urine and feces during the last 14 days in each case.

In preparation for analysis the samples of feces, urine, or feed were digested with concentrated sulfuric-nitric acid mixture, and then made up to volume. Aliquots of these solutions were used to determine calcium by the McCrudden ('10; '11) method, and phosphorus by the Fiske and Subbarow ('25) method, using a photoelectric technique for the latter.

Following period IV the rats were gassed and the stomach, small intestine, and cecum were removed. The pH values of the contents of various segments were determined using a Beckman pH meter fitted with a glass electrode. Following this the small intestines and

[5] Cerelose.
[6] Crisco.
[7] Labco, no. 15–42.

cecum were freed of contents, washed, and ground in water containing chloroform. After autolysis, filtering, and dialysis, extracts were obtained, almost free of inorganic phosphorus, which contained an enzyme capable of splitting sodium phytate with the production of orthophosphate. This hydrolysis was accomplished by using 1 ml. of substrate (approximately 1% sodium phytate), 2 ml. of buffer (sodium diethylbarbiturate plus hydrochloric acid) at pH 7.7, and 1 ml. of enzyme extract. These solutions were analyzed for inorganic phosphorus at zero hours and after 20 hours at 38°C.

RESULTS

In all four periods the rats showed no important differences between groups in the rate of body gain; but, as was expected, those in periods III and IV gained more because of the increased length of periods. The average weights of the rats, at the beginning of the basal collections, were from 97 to 101 gm.; and at the end of the higher phosphorus collection periods from 129 to 135 gm. for the shorter period, and from 148 to 152 gm. for the longer period.

Average balances of calcium and phosphorus are given in table 1. The percentages of calcium and phosphorus representing direct retention, and the percentages of calcium and phosphorus in the urine and feces, are given in table 2. Also given in table 2 are the percentages of "utilization" of calcium and phosphorus, using the Kinsman et al. ('39) formula. Table 2 also shows the weights of phosphorus gained in bone and soft tissue (98% of the calcium retained is in the bone, and this value is 2.15 times the weight of phosphorus in the bone). The weight of phosphorus retained in the bone was less for the phytin rats.

The average pH values of the contents of different parts of the alimentary tracts of the disodium phosphate rats were: stomach, 5.85; upper small intestine, 5.97; middle small intestine, 6.26; lower small intestine, 7.28; and cecum, 6.39. The corresponding values for the phytin rats were 5.53, 6.07, 6.38, 7.35, and 6.52. The average pH for the small intestine alone was 6.50 for the disodium phosphate rats and 6.60 for the phytin rats.

In the enzyme experiment, the freed inorganic phosphorus divided by the available phytate phosphorus was taken as the fraction hydrolzyed. The hydrolysis varied widely between 3.8% and 21.7%. Phytase was present in the extracts of both the disodium phosphate rats and the phytin rats, but no differences were noted between the groups.

TABLE 1

Average balances of calcium and phosphorus.

NUMBER OF RATS	RATION	INTAKE		OUTPUT						RETENTION		
				Urine		Feces		Total				
		Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca/P
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Period I												
Nos. 1–12	Basal	134.0	61.0	28.0	1.5	32.2	16.5	60.2	18.0	73.8	43.0	1.7
Nos. 13–24	Basal	134.0	61.0	25.6	1.6	35.6	19.7	61.2	21.3	72.8	39.7	1.8
Nos. 25–36	Basal	134.0	61.0	25.5	1.7	37.1	19.5	62.7	21.1	71.3	39.9	1.8
Period II												
Nos. 1–12	Basal + Na_2HPO_4	219.6	125.0	6.8	9.5	102.7	31.5	109.4	41.0	110.2	84.0	1.3
Nos. 13–24	Basal + lecithin	219.8	123.0	29.3	1.1	91.0	39.3	120.0	40.4	99.8	82.6	1.2
Nos. 25–36	Basal + phytin	218.8	115.5	44.6	1.2	94.3	47.6	138.9	48.8	80.7	66.7	1.2
Period III												
Nos. 37–50	Basal	255.0	120.0	50.4	2.9	48.8	20.5	99.2	23.4	155.8	96.6	1.6
Nos. 50–64	Basal	253.7	119.4	53.0	2.8	47.1	20.2	100.0	23.0	153.7	96.4	1.6
Period IV												
Nos. 37–50	Basal + Na_2HPO_4	461.0	241.0	3.8	38.0	163.3	52.1	167.2	89.9	293.8	151.1	2.0
Nos. 50–64	Basal + phytin	508.0	238.6	38.5	4.9	185.1	86.8	223.6	91.6	284.4	147.1	2.0

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DISCUSSION OF RESULTS

The rats of all three groups on the basal ration, period I, had approximately the same direct retention of calcium and phosphorus. On the higher phosphorus rations, period II, the rats on the disodium phosphate ration and on the lecithin ration showed a higher direct retention of 67.2% of the dietary phosphorus, while the rats on the phytin ration showed only 57.8% phosphorus retention. The corresponding calcium retentions for period II were 50.2%, 45.4%, and 36.9%, respectively. In terms of the Kinsman et al. ('39) formula for "utilization", the rats on the disodium phosphate ration showed 63.9%, those on the lecithin ration 69.2%, and those on the phytin ration 49.4% phosphorus utilization. The corresponding calcium utilizations were 41.9%, 32.0%, and 16.1%, respectively.

On the higher phosphorus ration, period IV, the rats on the disodium phosphate ration showed a direct retention of 62.7% and those on the phytin ration 61.7% of phosphorus. The corresponding retentions for calcium were 63.7% and 56.0%, respectively. The "utilization" of disodium phosphate phosphorus was 45.0% and of phytin phosphorus 42.5%. The corresponding "utilizations" for calcium were 67.0% and 51.5%, respectively. The low utilization of the extra disodium phosphate phosphorus is attributed to a lower intake of calcium, for this ration, which made calcium the limiting element. The higher amount of phosphorus in the urine of the rats on the disodium phosphate ration is attributed to the use of phosphorus to eliminate extra sodium.

The percentage of phosphorus "utilized" from phytin is much less than that directly retained. This percentage of "utilization" more nearly represents the phytin phosphorus, whereas, the direct retention represents both the phosphorus of the basal part of the diet and of the supplementary phytin.

The "utilization" of calcium and of phosphorus varied much from rat to rat, which accounts for the large standard errors shown in table 2. These variations in "utilization" are probably influenced by differences in the mineral saturation of the rat bodies, both at the beginning and at the end of the experimental period.

The larger amount of phosphorus in the feces of the rats on the phytin rations signifies the poor utilization of phytin phosphorus. That the calcium did not affect the utilization of phosphorus by forming calcium phosphate is shown by the fact that with the same amount of calcium and phosphorus available both the disodium phosphate rats and the lecithin rats retained the phosphorus as well in period II as in the basal period I and better than did the phytin rats in period II.

TABLE 2

Average retention and utilization of calcium and phosphorus and distribution of phosphorus.[1]

NUMBER OF RATS	RATION	RETENTION		UTILIZATION		IN URINE		FECES		PHOSPHORUS GAIN		
		Ca	P	Ca	P	Ca	P	Ca	P	In bone	In soft tissue	
											Total	Per gram
		%	%	%	%	%	%	%	%	*mg.*	*mg.*	*mg.*
Period I												
Nos. 1–12	Basal	55.1 ± 1.5	70.6 ± 1.4	.....	.....	20.9	2.5	24.1	27.0	33.6	9.4	1.0
Nos. 13–24	Basal	54.3 ± 2.0	65.1 ± 2.0	.....	.....	19.1	2.7	26.6	32.3	33.2	7.3	1.0
Nos. 25–36	Basal	53.2 ± 1.3	65.4 ± 2.1	.....	.....	19.1	2.8	27.6	31.9	32.5	7.5	0.9
Period II												
Nos. 1–12	Basal + Na_2HPO_4	50.2 ± 2.1	67.2 ± 2.4	49.1 ± 5.2	63.9 ± 4.5	3.1	7.6	46.9	25.2	50.2	33.8	3.2
Nos. 13–24	Basal + lecithin	45.4 ± 1.4	67.2 ± 2.0	32.0 ± 4.1	60.2 ± 3.9	13.3	0.9	41.3	31.9	45.5	37.1	2.9
Nos. 25–36	Basal + phytin	36.9 ± 2.8	57.8 ± 3.0	16.1 ± 4.6	49.4 ± 7.0	20.4	1.2	43.1	41.2	36.8	29.9	2.9
Period III												
Nos. 37–50	Basal	61.1 ± 1.6	80.5 ± 1.2	.....	.....	19.8	2.4	19.2	17.1	69.5	29.0	1.1
Nos. 50–64	Basal	60.5 ± 1.3	80.8 ± 1.0	.....	.....	20.8	2.4	18.5	16.9	70.4	26.0	1.0
Period IV												
Nos. 37–50	Basal + Na_2HPO_4	63.7 ± 2.1	62.7 ± 1.4	67.0 ± 5.3	45.0 ± 2.9	0.9	15.8	35.4	21.6	133.3	18.8	1.1
Nos. 50–64	Basal + phytin	56.0 ± 2.3	61.7 ± 2.7	51.5 ± 5.1	42.5 ± 4.9	7.6	2.1	36.4	36.3	128.0	23.2	1.2

[1] Plus and minus values are the standard errors of the means.

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The higher average pH value of the intestinal contents of the phytin rats was associated with the higher average amount of phosphorus in the feces of these rats. The fact that the calcium content of the feces of both groups of rats was about the same, whereas, the fecal phosphorus of the phytin group was the higher, suggests that the higher intestinal pH was not optimal for the activity of the enzyme phytase. This would tend to limit the amount of phytin phosphorus available for absorption. This effect would be in addition to and not in place of the well-known effect of pH on the solubility of calcium-phosphorus salts. The higher intestinal pH of the phytin rats is in agreement with the higher pH values for rats on a rachitogenic ration reported by Eastman and Miller ('35).

The detection of an enzyme, phytase, in the extracts of the intestinal wall in both groups of rats agrees with the findings of Patwardhan ('37). However, there was no correlation between enzyme activity and utilization of phytin phosphorus. Such a correlation would not necessarily exist since the utilization of phytin phosphorus would be affected by conditions other than the freeing of this phosphorus from its organic combination.

SUMMARY

The utilization of phytin, lecithin and disodium phosphate phosphorus by growing albino rats was studied by means of balance experiments, efficiency of utilization being expressed as the difference in retention divided by the difference in intake.

In one experiment disodium phosphate phosphorus and crude soy bean lecithin phosphorus were utilized to the extent of 63.9% and 69.2%, respectively. Phytin phosphorus was utilized to the extent of 49.2%. The corresponding calcium utilizations were 41.9, 32.0, and 16.1%, respectively.

In another experiment of longer duration the disodium phosphate phosphorus was utilized to the extent of 45.0%, and the phytin phosphorus to the extent of 42.5%. This low utilization of disodium phosphate phosphorus was apparently due to a low level of calcium in the ration which made calcium the limiting element instead of phosphorus. The corresponding calcium utilizations were 67.0 and 51.5%, respectively.

The higher amount of phosphorus in the feces of the phytin rats apparently signified lower absorption.

The pH values for intestinal contents of individual rats varied much, but the average pH value, 6.60, for the phytin rats was higher than

that, 6.50, for the disodium phosphate rats. This higher average pH value for the intestinal contents of the phytin rats was associated with a lower utilization of phytin phosphorus.

The intestinal wall extracts for both the disodium phosphate rats and the phytin rats showed the presence of a phytate-splitting enzyme. No correlation was found between the enzyme activity of the extracts and the utilization of phytin phosphorus. However, the pH of the intestine may influence the activity of the phytase.

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Lecithins and Lysolecithins of Wheat Flour[1]

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Abstract

Lecithin and lysolecithin from the bound lipid of Thatcher wheat endosperm were separated and purified by column and thin-layer silicic acid chromatography. Lecithin was hydrolyzed with phospholipase *A* (*Crotalus adamanteus*) and the products isolated and purified by silicic acid chromatography. The fatty acid composition of the original lecithin and lysolecithin and of the hydrolysis products was determined by gas-liquid chromatography.

The fatty acids in the beta position of the lecithin were found to be almost entirely unsaturated, whereas those in the alpha position were saturated and unsaturated in nearly equal amounts. The differences between the fatty acid composition of the original lysolecithin and that of the lysolecithin obtained by hydrolysis of lecithin with phospholipase *A* suggested the presence of both alpha and beta acyl species in the naturally occurring lysolecithin.

Introduction

THE STRUCTURE OF LECITHINS and their fatty acid distribution have been studied by hydrolysis with phospholipase *A* (1). Earlier work suggested that the ester bond with unsaturated fatty acid was hydrolyzed more readily (2,3). More recent work (4-8) has shown that phospholipase *A* specifically hydrolyzes the ester bond at the beta position. The large proportion of unsaturated fatty acids found in the beta position had given rise to the earlier conclusion that unsaturated acids were preferentially liberated. Definite positional specificity has been established by de Haas and van Deenen (4) who found that only stearic acid was released when synthetic α oleoyl β stearoyl lecithin was hydrolyzed by phospholipase *A* from snake venom (*Crotalus adamanteus*). Thus phospholipase *A* hydrolysis can be used to determine the positional distribution of fatty acids in lecithin.

The lipids of wheat endosperm have been studied in this laboratory. The free and bound lipids from three different types of wheat were separated into their component classes and the differences within and between classes studied (9). The components of the phospholipids and glycolipids were also separated and compared (10). A study of the fatty acid distribution in lecithin from Thatcher wheat will be reported in this paper.

Experimental and Results

Extraction

Flour was milled (9) from Thatcher wheat. The extraction was carried out in two stages. The free lipids were extracted by shaking 20 g flour with 180 ml cold (1C) deoxygenated hexane and centrifuging at 5 to 15C. The bound lipids were released by shaking the hexane-extracted residue with 180 ml water saturated butanol for 1 hr at 1C in stoppered centrifuge bottles. The suspension was centrifuged for 1 hr at below 15C and the solvent removed from the extract in a rotary evaporator at below 35C. The lipid was kept under nitrogen atmosphere at all times.

Purification and Separation of Lipid Components

The lipid thus extracted was purified by passage through a column of Sephadex (11) to remove non-lipid contaminants. The purified lipid was fractionated by means of silicic acid column chromatography as described by McKillican and Sims (9). The lecithin- and lysolecithin-rich fractions were further purified by TLC using chloroform-methanol-water (65:25:2) as developing solvent. The lipid was then eluted from the silica gel with chloroform-methanol (1:1).

Phospholipase A Hydrolysis

Crotalus adamanteus venom (Ross Allen Reptiles) provided the phospholipase *A* used in this study. Hydrolysis of the lecithin was carried out essentially according to Long and Penny (12). The venom was dissolved in 0.005 M aqueous calcium chloride in the proportion of 1 mg/ml. Fifty microliters of this solution was added to 6 to 8 mg of lecithin in 5 ml of diethyl ether. The hydrolysis was carried out with continuous shaking under nitrogen atmosphere, and was essentially complete in 21 hr. The time necessary for completion of the reaction was determined by monitoring with thin-layer chromatography (TLC). The aliquots of the hydrolysis mixture taken for TLC were developed with chloroform-methanol-water (65:25:2) and the spots revealed by iodine vapor (13) or Dragendorff reagent (14). The reaction was considered complete when no lecithin was detected. When the hydrolysis was complete the mixture was evaporated to dryness under nitrogen and the residue dissolved in anhydrous deoxygenated chloroform. The reaction products were separated on a 1.0 cm I.D. column containing 2 g of 325 mesh silicic acid (Bio-Rad Laboratories). Fractionation was accomplished by solvents of increasing polarity from pure chloroform to pure methanol (15 ml of 100:0, 30 ml of 75:25, 30 ml of 50:50, 15 ml of 25:75, 45 ml of 0:100). The free fatty acids from the column were methylated (15) and the methyl esters and lysolecithin were purified by means of TLC and weighed. The recovery of methyl esters of fatty acids was 3.9 mg per 10 mg lecithin (theoretical 3.7 mg) and of lysolecithin 6.6 mg per 10 mg lecithin (theoretical 6.7 mg).

Fatty Acid Composition

The original lecithin and lysolecithin as well as hydrolysis products were transesterified with boron trifluoride in methanol as described by Morrison (15). The methyl esters were purified by developing with hexane-diethyl ether-acetic acid (90:10:1) on thin-layer plates. The purified methyl esters were eluted from the silica gel with chloroform-methanol and analyzed by gas-liquid chromatography (GLC). A column 6 ft long containing 5% DEGS on Gas Chrom Q was used at 178C in a Research Specialties series 600 gas chromatograph operating with a flame ionization detector. Percentages were calcu-

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lated from calibration curves obtained by the use of an internal standard. As shown in Table I, the principal fatty acids were palmitic, oleic and linoleic acids. The fatty acid composition of the beta-position in lecithin was determined by the fatty acids obtained by hydrolysis. The unsaturated fatty acids amounted to 96.9% and linoleic alone contributed more than 75%. The fatty acid composition of the alpha position was determined in two ways: (a) by difference between the beta position and the total composition of the original lecithin; and (b) by the analysis of the hydrolyzed lysolecithin. As determined by both methods, saturated and unsaturated acids were present in approximately equal amounts. Palmitic acid was the largest single component.

Discussion

The beta position of the lecithin was occupied primarily by linoleic acid. In the alpha position there was slightly more palmitic than linoleic.

The total saturated fatty acid in the beta position was 3.1%, thus the maximum beta-saturated species, i.e. SS + US, in lecithin was 3.1% (US = α unsaturated, β saturated). Similar fatty acid distribution has been found in other plant lecithins. Privett (16) found no SS in soybean lecithin and only a trace in safflower lecithin. In the alpha position the total saturated fatty acid was 59.2% thus the SU species was between 56.1 and 59.2% (SU = β unsaturated, α saturated). Similarly the UU species amounted to between 37.7 and 40.8% of which up to 36.5% could be dilinoleoyl lecithin. A relatively high proportion of UU species is characteristic of plant lecithins; animal lecithins, by contrast, have much lower UU, 2 to 10%.

Lysolecithin was shown by TLC to be present in the original wheat endosperm extract before column separations were carried out, and thus was primarily naturally occurring and not a product a transmethylation by methanol on the silicic acid column. The native lysolecithin contained more linoleic and less palmitic than the alpha position of lecithin. Moreover, the native lysolecithin contained 2% linolenic acid whereas none was found in the alpha position of lecithin. Assuming that the fatty acids occur in the same position in native lysolecithin as in lecithin, these differences suggest that the native lysolecithin is a mixture of alpha and beta acyl species. Both species were found by Tattrie (8) in animal lysolecithin. Van den Bosch (17) studied the fatty acid distribution of rat liver lecithin, naturally occurring lysolecithin and lysolecithin produced by phospholipase A hydrolysis. He found differences indicating that the beta species amounted to 35% of the naturally occurring lysolecithin.

The lysolecithin in the endosperm lipids of Thatcher wheat thus appears to be a naturally occurring constituent, made up of both alpha and beta species.

TABLE I

Fatty Acid Composition
Mole %

Fatty acid	Original		Hydrolyzed lecithin	
	Lecithin	Lysolecithin	Alpha position[a]	Beta position[b]
16:0	25.7	43.7	55.0	3.1
18:0	0.7	0.8	4.2	0
18:1	11.1	5.4	4.3	15.4
18:2	60.5	48.1	36.5	77.4
18:3	2.0	2.0	0	4.1

[a] Lysolecithin obtained by hydrolysis.
[b] Free fatty acids obtained by hydrolysis.

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ABSORPTION AND METABOLISM OF LECITHIN AND LYSOLECITHIN BY INTESTINAL SLICES

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SUMMARY

1. Intestinal absorption and metabolism of lysolecithin and lecithin has been studied using hamster and rat intestinal slices and inverted sacs, and [*choline-Me*-^{3}H]- [*acyl*-^{3}H]lysolecithin and lecithin as substrates.

2. The physicochemical form of the substrates has been, for lecithin, either a liquid crystalline dispersion in buffer or mixed lecithin–sodium taurodeoxycholate micellar solutions, and for lysolecithin, either a micellar solution in buffer or mixed lysolecithin–sodium taurodeoxycholate micellar solution. The classification of the physicochemical form of the substrates has been derived from their behaviour on gel filtration.

3. Lysolecithin is well absorbed and metabolized mainly to lecithin.

4. The uptake and metabolism of lysolecithin is not influenced by the presence of sodium taurodeoxycholate in the medium.

5. The absorption and metabolism of lysolecithin is the same in slices from all levels of the small intestine except the most distal part.

6. No definite evidence could be obtained that lecithin is absorbed intact.

INTRODUCTION

It has long been assumed that phospholipids are hydrolyzed in the intestinal tract during absorption, and enzymes capable of splitting phospholipids have been found in many tissues including pancreas, pancreatic juice and intestinal mucosa. ARTOM AND SWANSON[1] studied the absorption of ^{32}P-labeled phospholipids in rats and found evidence for the absorption of ^{32}P partly as the intact phospholipid molecule and partly as $^{32}P_i$ or glycerophosphate.

BLOMSTRAND[2] obtained evidence for some absorption of intact phospholipids by feeding doubly labeled phospholipids to rats with a thoracic duct fistula. He found that when feeding acyl-labeled phospholipids, a much larger part of the active fatty acid was obtained in the phospholipids of thoracic duct lymph than when feeding

The lecithin was isolated by silicic acid chromatography[11]. The lysolecithin–cadmium chloride complex was prepared from egg lysolecithin. [α-*Palmitoyl*-^{3}H] lysolecithin was prepared from [$^3H_2$]palmitoyl lecithin as described above for lysolecithin. Radioactive purity was checked by thin-layer chromatography, and was better than 95% (ref. 18). [*Choline-Me*-^{3}H]lecithin: ^{3}H-labeled methyl choline was injected intraperitoneally into a rat fed a choline-free diet for 10 days. About 20h after injection the rat was killed, the liver was homogenized and extracted with chloroform–methanol, 2:1. The lecithin was isolated by silicic acid chromatography[11]. [α-*Choline-Me*-^{3}H]lysolecithin was prepared from [*choline-Me*-^{3}H]lecithin as described above for lysolecithin. [*Choline-Me*-^{3}H]-glycerylphosphorylcholine was prepared from [*choline-Me*-^{3}H]lecithin[9].

EXPERIMENTAL

Gel filtration experiments

The gel was swollen in 0.15 M NaCl solution and packed in columns with a diameter of 16 mm and with a total gel volume of 50–60 ml. When the column was to be eluted with a solution other than 0.15 M NaCl, it was pre-equilibrated by running at least 2 column volumes of the actual solution through the column. The columns were run at room temperature with a drop flow of 10–20 ml/h. A drop counter was used, but because of variations in drop size (depending on variations in surface tension) the fractions were also weighed in preweighed test tubes. Generally, fractions close to 1 ml were collected[14]. The solution to be studied (volume 1 ml) was applied to the column and the column then eluted with about 70 ml.

TABLE I

GEL FILTRATION ON SEPHADEX G-100 COLUMNS OF LECITHIN OR LYSOLECITHIN ALONE OR IN COMBINATION WITH BILE SALT AND MONOOLEIN

The sodium taurodeoxycholate concentration used was 6 μmoles/ml. Lecithin in 0.15 M NaCl forms a liquid crystalline state with large aggregates, which are completely excluded from the inner volume of Sephadex G-100 (ref. 1). Lysolecithin in 0.15 M NaCl forms a micellar solution with an aggregate size somewhat below 100000 (refs. 6, 7). When sodium taurodeoxycholate is added, mixed bile salt–phospholipid micelles are formed, which are somewhat larger than the pure sodium taurodeoxycholate micelles[2,3,8,9]. Monoolein and phospholipids in a bile salt solution were eluted in the same peak indicating the formation of mixed monoolein–phospholipid–bile salt micelles somewhat larger than the pure bile salt micelles.

Expt.	*Sample applied*	*Eluant*	K_{av}	*Physical form*	*Aggregate weight*
1	Lecithin (2 or 10 μmoles)	0.15 M NaCl	0	Liquid crystalline	100000
2	Lecithin (2 μmoles)	0.15 M NaCl + sodium taurodeoxycholate	0.45	Mixed micellar	15000
3	Lecithin (10 μmoles)	0.15 M NaCl + sodium taurodeoxycholate	0.30	Mixed micellar	30000
4	Lecithin (2.5 μmoles + monoolein, 2.5 μmoles)	0.15 M NaCl + sodium taurodeoxycholate	0.40	Mixed micellar	25000
5	Lysolecithin (0.1 μmole)	0.15 M NaCl	—	Micellar	—
6	Lysolecithin (1.2 or 10 μmoles)	0.15 M NaCl	0.03–0.10	Micellar	80000–100000
7	Lysolecithin (10 μmoles)	0.15 M NaCl + lysolecithin	0.17	Micellar	80000
8	Lysolecithin (2 μmoles)	0.15 M NaCl + sodium taurodeoxycholate	0.45	Mixed micellar	15000
9	Lysolecithin (10 μmoles)	0.15 M NaCl sodium taurodeoxycholate	0.33	Mixed micellar	25000
10	Lysolecithin (2.5 μmoles + monoolein, 2.5 μmoles)	0.15 M NaCl + sodium taurodeoxycholate	0.48	Mixed micellar	15000

labeled free fatty acids. A considerable amount of activity, however, was also found in the neutral fat of the lymph, mainly as triglyceride. BLOMSTRAND concluded that an extensive hydrolysis of phospholipids occurs during absorption but that part of the phospholipid is absorbed as intact molecules.

BLOMSTRAND[2] also studied the effect of bile on the absorption of phospholipid by feeding acyl-labeled glycerophospholipid to rats with both the thoracic duct and the bile duct cannulated but with free passage for the pancreatic juice to the duodenum. He found that bile is not obligatory for the absorption of phospholipid fatty acids and that the absence of bile does not influence the distribution of activity into phospholipids and the neutral fat of the lymph. Without bile, however, only one-third of the absorbed phospholipid fatty acid was transported by the thoracic duct lymph as compared with the normal conditions.

One phospholipid-splitting enzyme of the pancreatic tissue is a phospholipase A capable of splitting off the β fatty acid of diacyl-phospholipids giving the corresponding α-lyso compound as the product[3]. Lecithin is the predominant phospholipid supplied to the intestine in the bile and in the diet, and α-lysolecithin is the predominant phospholipid specimen found in the intestinal lumen[4]. It has recently been demonstrated that pancreatic lipase splits off the 1-linked fatty acid of lecithin during formation of β-lysolecithin[5].

The intestinal mucosa contains enzymes capable of splitting the acyl bonds in both lysolecithin and lecithin[26]. The enzymes appear to be associated with ribosomal structures[6].

In the present experiments the intestinal absorption of lecithin and lysolecithin has been studied *in vitro* using rat and hamster intestinal slices and specifically labeled substrates. The physical form of the substrates has been, for lecithin, either a liquid crystalline dispersion in buffer or a mixed lecithin–sodium taurodeoxycholate micellar solution, and for lysolecithin, either a micellar solution in buffer or a mixed lysolecithin–sodium taurodeoxycholate micellar solution. The basis for this classification was derived from gel filtration experiments.

MATERIALS

Sephadex G-100 (TO 5218) was obtained from Pharmacia, Uppsala. Sodium taurodeoxycholate was synthesized according to the method of NORMAN[7] as modified by HOFMANN[8]. The purity was better than 98%. Crude egg lecithin was obtained from the Vitrum Company, Stockholm. Glycerylphosphorylcholine–cadmium chloride complex was prepared from crude egg lecithin[9]. [*Oleoyl*-9,10-$^3H_2$] lecithin and [*Palmitoyl*-9,10-$^3H_2$] lecithin were prepared by acylation of the glycerylphosphorylcholine–cadmium chloride complex with the acid chlorides[10]. The lecithin was purified by silicic acid chromatography[11]. α-Lysolecithin: 100 mg pure egg lecithin was emulsified in 3 ml phosphate buffer (pH 7.0) containing 2 mg bee venom phospholipase A. 75 ml ether was added, the mixture then being shaken vigorously and stored at room temperature for at least 3 h[12]. Lysolecithin was isolated by 3 extractions using ether–petroleum–ethanol–phosphate buffer (pH 6.3; 3:3:3:1:1, by vol.). The lysolecithin was extracted from the combined lower phase with chloroform. The purity of the product was checked by thin-layer chromatography. [*β-Oleoyl*-9,10-^3H] lecithin was prepared by acylation of lysolecithin–cadmium chloride complex with oleic acid chloride[13].

To indicate the void volume, blue dextran with a molecular weight of 500000 was used. The concentration of blue dextran was measured at 600 mμ. Radioactivity was determined by liquid scintillation counting using a Packard Spectrometer and a dioxan–naphthalene solution (1 ml of eluate + 10 ml scintillation fluid)[15].

The types of experiments performed are shown in Table I. In expt. 7 the column was eluted and pre-equilibrated with a labeled lysolecithin solution having the same specific activity as the sample and with a concentration well above the critical micellar concentration of lysolecithin. Assuming that the concentration of molecularly dispersed lysolecithin is the same at any concentration above the critical micellar concentration, the lysolecithin micelles of the sample meet the same environment of molecularly dispersed labeled lysolecithin as in the sample when passing the column, and the effects of the rapid equilibrium between micellar and molecularly dispersed lysolecithin are eliminated.

Incubation experiments

(a) *Preparation of incubation medium.* Labeled lecithin or lysolecithin were stored in dilute chloroform solution in the cold. The chloroform was evaporated from aliquots of these solutions and the desired amounts of Krebs-Ringer and sodium taurodeoxycholate solutions were added and the lecithin or lysolecithin dispersed by agitation. Finally the solutions were adjusted to the concentrations desired in the different experiments by adding Krebs–Ringer buffer (pH 6.3) with or without sodium taurodeoxycholate. The solutions containing lecithin or lysolecithin in sodium taurodeoxycholate were always clear as were the solutions of lysolecithin in buffer. Dispersions of lecithin in buffer without bile salt were turbid.

(b) *Tissue preparation.* Intestinal slices were prepared from everted hamster or rat small intestine by the procedure of AGAR, HIRD AND SIDLEN[16] and JOHNSTON AND BORGSTRÖM[17]. Inverted sacs were prepared by tying the ends of a 2.5-cm long piece of gut.

(c) *The incubations* were carried out in 1-ml solution containing 150–200 mg wet weight of intestinal slices or sacs and 10 μmoles glucose/ml. All solutions were gassed with oxygen for approx. 1 h prior to addition of the tissue and the incubations were carried out at 37° in a reciprocating shaker for 1 h under oxygen.

(d) *Extracting procedure.* Following the incubation, the incubation medium was poured off and the slices were washed twice with 1 ml of 0.15 M NaCl containing 2.4 μmoles of sodium taurodeoxycholate per ml. The washings were combined and extracted with 18 ml chloroform–methanol (2:1, by vol.). The intestinal slices were homogenized in 3 ml chloroform–methanol (2:1, by vol.) the tube was washed twice with 3.5 ml chloroform–methanol (2:1, by vol.) and the combined washings and homogenate were equilibrated against 4 ml 0.15 M phosphate buffer (pH 6.3). The upper phases were withdrawn and the chloroform phase was dried with water-free Na_2SO_4, filtered and evaporated to dryness. 5–6% of the lysolecithin is lost to the upper phase in this extraction procedure.

(e) *Separation of the lipids.* The extracted lipids were dissolved in 1 ml chloroform. 100 μl was taken directly for scintillation counting; 100 μl was resolved by thin-layer chromatography on silicica gel-G plates using chloroform–methanol–acetic acid–water (50:25:8:4, by vol.)[18]. The spots were scraped off and eluted with 4 ml chloroform–methanol–formic acid–water (9:9:1:1, by vol.) into the scintillation flask.

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the mixture being blown to dryness with air. The lysolecithin spots were eluted with 4 ml chloroform methanol formic acid water (4:14:1:1, by vol.) and then handled in the same way as the lecithin. For the separation of the neutral lipids and fatty acids another 100-μl sample was applied to the same type of silicic gel plate and chromatographed in hexane ether-methanol-acetic acid (90:20:3:2, by vol). Liquid scintillation counting was performed using a Packard spectrometer and dioxane naphthalene solution[14].

Types of slice incubations performed

Substrate	*Medium*	*Type of slices*
A. ^3H Oleic acid		
a) 1 μmole	Krebs–Ringer buffer + 2.4 μmoles sodium taurodeoxycholate	Sliced from the upper third of the small intestine
b) 1 μmole	Krebs–Ringer buffer + 0.8 μmole lysolecithin	*ditto*
c) 1 μmole	Krebs–Ringer buffer	*ditto*
d) 1 μmole	Krebs–Ringer buffer + 2 μmoles lysolecithin	*ditto*
B. [Palmitoyl-^{3}H]lysolecithin		
a) 1 μmole	Krebs–Ringer buffer + 2.4 μmoles sodium taurodeoxycholate	Mixed from the upper third of the small intestine
2 μmoles	*ditto*	*ditto*
1 μmole + 1 μmole oleic acid	*ditto*	*ditto*
2 μmoles + 2 μmoles oleic acid	*ditto*	*ditto*
b) 2 μmoles	Krebs–Ringer buffer + 0–6 μmoles sodium taurodeoxycholate	*ditto*
c) 2 μmoles	Krebs–Ringer buffer	Slices from different levels of the small intestine
d) 2 μmoles	Krebs–Ringer buffer + 2.4 μmoles sodium taurodeoxycholate	*ditto*
C. [Choline Me-^3H]lysolecithin		
a) 1 μmole + 1 μmole oleic acid	Krebs–Ringer buffer + 2.4 μmoles sodium taurodeoxycholate	From the upper third of the small intestine
2 μmoles + 2 μmoles oleic acid	*ditto*	*ditto*
b) 2 μmoles	Krebs–Ringer buffer + 0–6 μmoles sodium taurodeoxycholate	From the upper third of the small intestine
c) 2 μmoles + 2 μmoles oleic acid	Krebs–Ringer buffer	Inverted sacs from the upper half of the small intestine
ditto	Krebs–Ringer buffer + 2.4 μmoles sodium taurodeoxycholate	*ditto*
Different incubation times 5–60 min.		
D. [Choline Me-^3H]–glyceryl phosphorylcholine		
2 μmoles	Krebs–Ringer buffer	From the upper third of the small intestine

Substrate	*Medium*	*Type of slices*
E. [^{3}H]Lecithins		
a) 1 μmole [*β-oleoyl-*^{3}H]-lecithin	Krebs-Ringer buffer	From the upper third of the small intestine
2 μmoles [*β-oleoyl-*^{3}H]-lecithin	*ditto*	*ditto*
1 μmole [*β-oleoyl-*^{3}H]-lecithin	Krebs-Ringer buffer + 2.4 μmoles sodium taurodeoxycholate	*ditto*
2 μmoles [*β-oleoyl-*^{3}H]-lecithin	*ditto*	*ditto*
b) 2 μmoles	*ditto*	Slices from rat fasted for 24 h
2 μmoles	*ditto*	Slices from rat without pancreatic flow into the intestine for 24 h
c) 2 μmoles [*α,β-dioleoyl-*^{3}H]lecithin	*ditto*	Mixed slices from the upper third of the small intestine
ditto	Krebs-Ringer buffer	*ditto*
d) 2 μmoles [*choline-Me-*^{3}H]lecithin	*ditto*	*ditto*
ditto	Krebs-Ringer buffer + 2.4 μmoles sodium taurodeoxycholate	*ditto*

RESULTS

Gel filtration experiments

Lecithin dispersed in 0.15 M NaCl was eluted with the void volume in a single peak (Fig. 1a). α-Acyl-lysolecithin under the same conditions was eluted in a single peak corresponding to a K_{av} (ref. 19) of 0.03–0.10 (Fig. 1b). With 10 μmoles applied to the column 90% of the active substance was recovered in this peak; with 2 μmoles, 70%, and with 1 μmole, 35%. A constant amount of activity per ml was present in all the fractions between the peak and V_t. The fractions after V_t contained smaller amounts of activity. When 0.1 μmole was used a low recovery was obtained with no definite peak. When the column was pre-equilibrated with a lysolecithin solution of the same specific activity and a concentration above the critical micelle concentration a K_{av} of 0.05 was obtained.

When eluted in sodium taurodeoxycholate–NaCl solution both lecithin and lysolecithin were eluted in a single peak with K_{av} values between 0.30 and 0.45 depending on the concentration of phospholipid in the sample applied to the column. The higher the phospholipid concentration the lower was the K_{av}.

When [^{3}H]lysolecithin or [^{3}H]lecithin was applied in a solution of bile salt containing [^{14}C]monoolein both the ^{3}H and ^{14}C activities were eluted in a single peak with K_{av} values between 0.40 and 0.48.

The results of the gel filtration experiments are shown in Table I. The aggregate weights have been calculated from the data of LAURENT[19] assuming a spherical form[27].

Intestinal slice and sac experiments

A. Oleic acid. When sodium taurodeoxycholate was present in the medium 37–70% of the activity was taken up per 150 mg slices. Without bile salt in the medium the uptake was 21–47% per 150 mg tissue. When no bile salt was present, the presence of lysolecithin in the medium did not influence the uptake (Fig. 2).

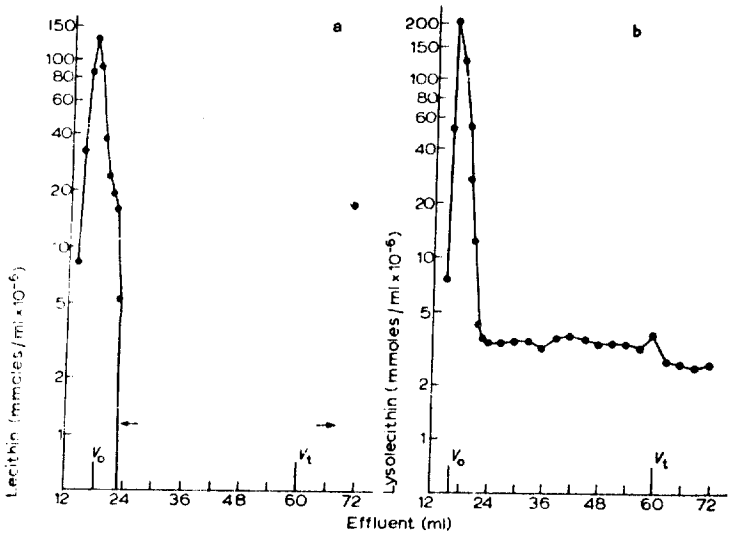


Fig. 1a. 1 μmole lecithin eluted on Sephadex G-100 in 0.15 M NaCl. The lecithin forms large liquid crystalline aggregates which are completely excluded from the inner volume of the gel, and no detectable amount of active lecithin is found in the fractions following the peak (between the arrows), indicating the absence of molecularly dispersed lecithin. The lecithin solution could therefore be classified as being non-micellar. 1b. 1 μmole lysolecithin eluted on Sephadex G-100 in 0.15 M NaCl. K_{av} of the peak volume containing lysolecithin micelles corresponds to that of a spherical molecule with a weight 80000–100000. The fraction following the peak volume contains molecularly dispersed lysolecithin. Thus lysolecithin in water could be classified as a micellar solution.

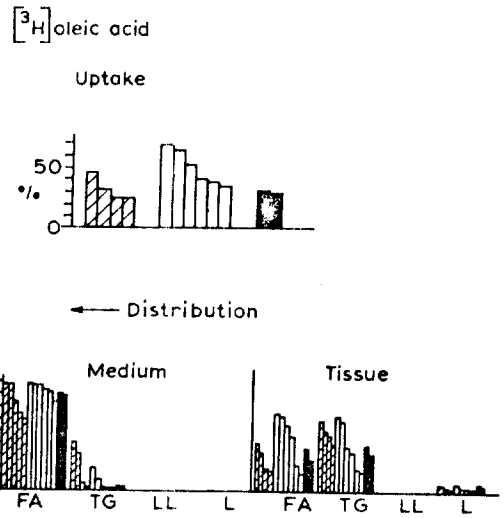
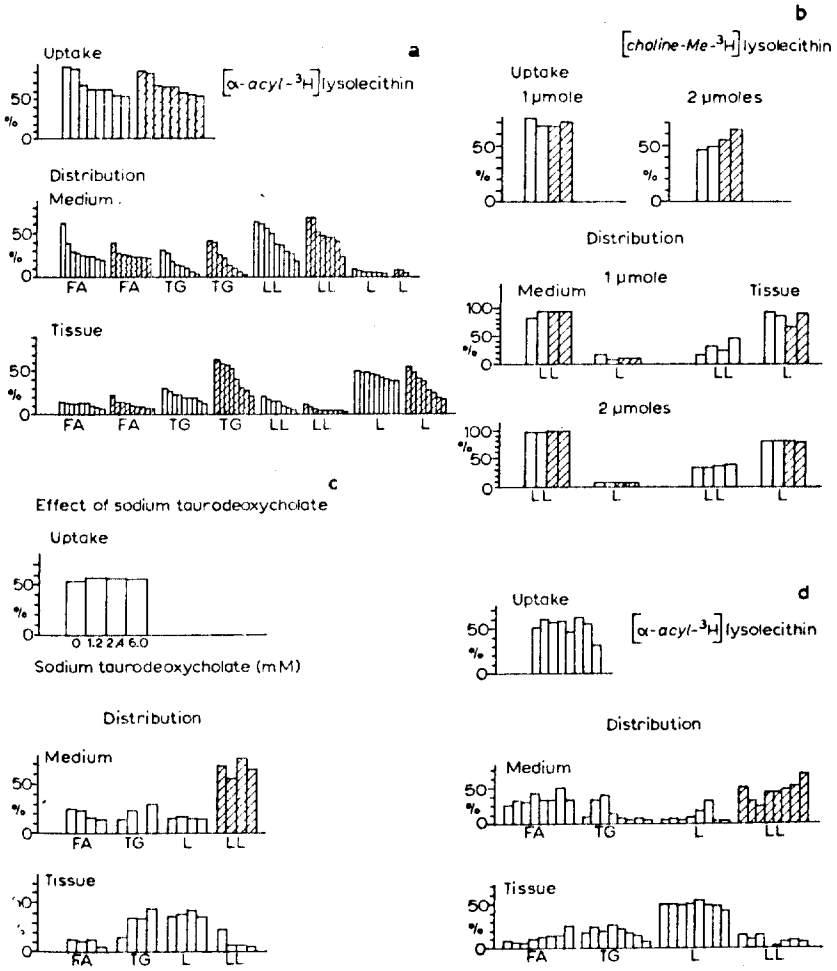


Fig. 2. % uptake per 150 mg tissue (upper) and distribution of activity in medium, (lower left) and tissue (lower right) after incubations of [^{3}H]oleic acid with intestinal slices. 4–6% of the absorbed fatty acid is incorporated into lecithin. One column is the result from one incubation. Hatched areas, no sodium taurodeoxycholate or lysolecithin; open areas, 2.4 μmoles sodium taurodeoxycholate; black areas, 2 μmoles lysolecithin. FA, fatty acid; TG, triglyceride; LL, lysolecithin; L, lecithin.



g. 3a. % uptake per 150 mg tissue (upper), and distribution of activity in the medium (middle) d tissue (lower) after incubating intestinal slices with 2 µmoles [α-*acyl*-³H lysolecithin. One lumn is the result from one incubation. Part of the lysolecithin is split by lysolecithinase from e mucosal cell, but a considerable part of it is absorbed intact and converted to lecithin by the ces. No influence of sodium taurodeoxycholate was observed. Each column is the result of one periment. The columns are in order of size. Open areas, no sodium taurodeoxycholate; hatched eas, 2.4 µmoles sodium-taurodeoxycholate. FA, fatty acid; TG, triglyceride; LL, lysolecithin; lecithin. 3b. % uptake per 150 mg tissue after incubating intestinal slices with 1 µmole (upper t) or 2 µmoles (upper right) [α-*choline-Me*-³H]lysolecithin and distribution of activity (1 µmole iddle figure; 2 µmoles: lower figure). 40–50% of the lysolecithin is split by lysolecithinase from e mucosa and the activity found in water-soluble substances. A considerable part of the lysolecithin, however, is absorbed intact and converted to lecithin by the slices, probably by a direct cylation. One column is the result from one incubation. The columns represent % activity still present in lipid form after incubation. Hatched areas, 2.4 µmoles sodium taurodeoxycholate; open areas, no sodium taurodeoxycholate. LL, lysolecithin; L, lecithin. 3c. % uptake per 150 mg tissue (upper) and distribution of activity in the medium (middle) and tissue (lower) after incubation of intestinal slices with [α-*acyl*-³H]lysolecithin in a medium containing different concentrations of sodium taurodeoxycholate. No influence of the bile salt was observed. FA, fatty acid; TG, triglyceride; L, lecithin; LL, lysolecithin. 3d. Uptake per 150 mg tissue (upper) and distribution of activity in the medium (middle) and tissue (lower) after incubation of slices from different levels of the small intestine with 2 µmoles α *acyl*-³H lysolecithin. No. 1 is the most proximal part; No. 8 the most distal. No significant differences in the uptake and conversion of lysolecithin were observed except for the low uptake in the most distal part. Each column is the result of one incubation. FA, fatty acid; TG, triglyceride; L, lecithin, LL lysolecithin

After incubation all activity in the medium, except small amounts as triglycerides, was found as free fatty acids. In the slices generally 20–65% of the activity was incorporated into triglycerides, 10–30% into free fatty acids and the remaining part into diglycerides, monoglycerides and lecithin. 4–6% of the activity of the slices was found in lecithin (Fig. 2). When 0.5–8 μmoles unlabeled lysolecithin was added to the medium, the incorporation into lecithin increased to 6–9% (Fig. 2).

B. Lysolecithin. With acyl label 48–74% of the activity was found in the slices per 150 mg tissue (Fig. 3a); of the ^{3}H choline label, 45–60% of activity still present in lipid form after incubation was found per 150 mg tissue (Fig. 3b). 40–50% was found as water-soluble substances. When the sodium taurodeoxycholate concentration was varied from zero to 6 μmoles per ml no difference in the uptake could be observed (Fig. 3c). The uptake was the same with slices from different parts of the small intestine except with slices from the most distal part (Fig. 3d). In the experiments with inverted sacs, 12–13% of the activity added was taken up by 150 mg tissue in 60 min. Addition of sodium taurodeoxycholate to the medium did not give any significant change in the uptake at any incubation time. In a sac with the serosa side against the medium a very small uptake was observed. When the sac had been heated to 100° for 1 min before incubation, good uptake but no acylation to lecithin was observed.

Distribution of activity. (1) [*α-Palmitoyl-*^{3}H]lysolecithin. After incubation 50–70% of the activity was found in the medium as lysolecithin. 15–25% was free fatty acid and generally rather small amounts were found as triglycerides, diglycerides, monoglycerides and lecithin. The relationship between lysolecithin and free fatty acid activities was the same whether sodium taurodeoxycholate was present or not, and it did not vary when slices from different levels were used (Fig. 3a, Table II).

In the slices, 22–64% of the activity was found in the triglyceride-fraction 21–59% in lecithin and the rest in diglycerides, monoglycerides and free fatty acids. Quite small amounts of activity were found as free fatty acids, when slices from the upper third of the intestine were used. In the distal part more activity was in free fatty acids, there being a corresponding decline in the triglyceride activity. The sum of triglyceride and free fatty acids activities was about the same for slices from all levels of the small intestine. No differences in the distribution of activity were observed when an equivalent or larger amount of unlabeled oleic acid had been added to the incubation medium (Figs. 3a, 3c, 3d, and Table II).

(2) [*Choline-Me-*^{3}H]lysolecithin. In the medium after incubation 78–94% of the lipid activity was in the lysolecithin fraction and the remaining part in the lecithin fraction. 40–50% of the activity added was lost to the upper phases when extracting with chloroform–methanol (2:1, by vol.). When acyl-labeled lysolecithin was used, the loss to the upper phase was negligible. The loss from the [*choline-*^{3}H]lysolecithin medium is probably due to the formation of [*choline-*^{3}H]glycerylphosphorylcholine due to lysolecithinase activity. When [*choline-Me-*^{3}H]glycerylphosphorylcholine was incubated, no activity was incorporated in lecithin or lysolecithin.

In the slices more than 70% of the activity was found in the lecithin fraction indicating a fairly complete acylation of the absorbed lysolecithin. The remaining activity was found as lysolecithin (Fig. 3b).

C. Lecithin. When [*β-oleoyl-*^{3}H]lecithin and [*α,β-oleoyl-*^{3}H]lecithin were incubated, varying uptakes were observed. The uptake varied between 7 and 36% when

TABLE II

The mucosal cells contain considerable lysolecithinase activity and during the incubations a large part of the fatty acid is split off and found as free fatty acid in the medium and as glyceride and free fatty acid in the tissue. The phospholipid activity after incubation is mainly in lecithin in the slices and in lysolecithin in the medium. Each figure is the result of one incubation.

[α-*Acyl*-^{3}H]*lysolecithin*	*Distribution of activity into phospholipids, glycerides and free fatty acids*			
	Phospholipids %		*(Glycerides + free fatty acids)* %	
[α-*Palmitoyl*-^{3}H]lysolecithin (2 μmoles) with sodium taurodeoxycholate	39 33 31 26	Mean, 32	61 67 69 74	Mean, 60
ditto + 2 μmoles oleic acid	50 40 37	Mean, 42	50 60 63	Mean, 58
[α-*Oleoyl*-^{3}H]lysolecithin μmoles with sodium taurodeoxycholate 2	61 53 47 42 32	Mean, 46	39 47 53 58 68	Mean, 54
ditto without sodium taurodeoxycholate	67 60 62 58 54 51 51 45 43	Mean, 55	33 34 38 42 46 49 49 55 57	Mean, 45
[α-*Palmitoyl*-^{3}H]lysolecithin 1 μmole with sodium taurodeoxycholate + 1 μmole oleic acid	53 45 43 32	Mean, 43	47 55 53 68	Mean, 57
ditto without oleic acid	39 34 33 32	Mean, 34	61 66 67 68	Mean, 66

no bile salt was present and between 7 and 63% when sodium taurodeoxycholate was added to the medium (Fig. 4). In two experiments with [^{3}H]choline-lecithin only 3–5% of the total activity was associated with the slices when the incubation was performed in Krebs–Ringer buffer and 8–10% when sodium taurodeoxycholate was added to the medium. When acyl-labeled lecithin was used, widely differing proportions of activity in the lecithin and free fatty acid fraction were observed in the medium after incubation. Only very small amounts of lysolecithin activity were found in the medium. In the experiment with the highest uptake, 15–47% of the activity in the slices was in the lecithin fraction when [β-*acyl*-^{3}H]lecithin was used and 27–73% when [α,β-*diacyl*-^{3}H]lecithin was used. (Fig. 4)

When the label was in the choline methyl group, 86–91% of the activity of the medium was in the lecithin fraction and the rest in the lysolecithin fraction. In

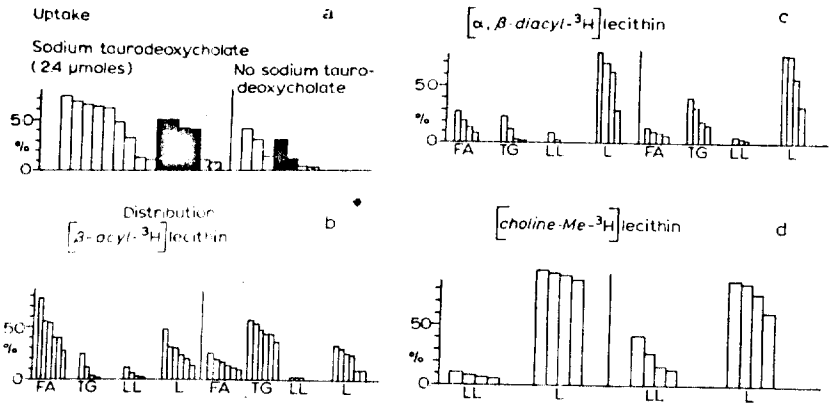


Fig. 4a. % uptake per 150 mg tissue after incubating intestinal slices with [*β-acyl*-^{3}H]lecithin, [*α,β-acyl*-^{3}H lecithin and [*choline-Me*-^{3}H]lecithin. Very varying uptake and distribution of activity was observed probably due to varying lecithinase activity in the medium. No definite evidence that lecithin was absorbed intact could be obtained. Open areas, [*β-acyl*-^{3}H]lecithin; black areas, [*α,β-diacyl*-^{3}H]lecithin; hatched areas, [*choline-Me*-^{3}H]lecithin. Fig. 4b, c and d show the distribution of activity after incubations with, respectively, [*β-acyl*-^{3}H]-, [*α,β-acyl*-^{3}H]-, and [*choline-Me*-^{3}H lecithin (medium: left; tissue: right). Each column is the result of one incubation. FA, fatty acid; TG, triglycerides; LL, lysolecithin; L, lecithin.

the slices 75–80% of the activity was found as lecithin; 20–25% as lysolecithin (Fig. 4).

When slices from a rat from which the pancreatic duct had been ligated 24 h before the experiment were incubated with [*β-acyl*-^{3}H]lecithin, 40–60% of the activity in the medium after incubation was in free fatty acid and 15–30% in lecithin. In the slices 12–18% of the activity was in lecithin and the rest in neutral fats. The uptakes were 25–55% per 150 mg tissue.

DISCUSSION

Lecithin and lysolecithin have characteristic bipolar properties due to the presence in the same molecule of the hydrophobic fatty acyl chain(s) and the hydrophilic phosphorylcholine group. Owing to this structural characteristic, lecithin can be easily dispersed in water forming a turbid solution. The solubility of lecithin with long-chain fatty acids in water, however, is extremely low. Lecithin dispersion in water therefore has to be characterized from the physicochemical point of view as liquid crystalline, with an aggregate weight of several millions[20]. In line with this, a dispersion in water passed the Sephadex G-100 columns with the void volume, *i.e.*, the aggregates were too big to penetrate the interior of the gel. No activity was found following the initial peak nor corresponding to the total volume of the column, indicating the very low molecular solubility of lecithin in water. In fact this behaviour on the gel column would characterise the lecithin dispersions as being non-micellar as, according to definition, a micellar solution should contain aggregates in equilibrium with a molecular dispersed phase.

When lecithin was dispersed in bile salt solution, clear solutions were obtained and the lecithin was eluted from the column with a K_{av} close to that earlier found for

the bile salt micelle[14]. It therefore seems clear that lecithin, in its capacity as an amphiphile[21], forms mixed micelles with the bile salt. If another amphiphile, monoolein, is added to the system, a mixed micelle with 3 components is formed and the lecithin and monoolein are eluted together in a symmetrical peak. The aggregate weight of the mixed bile salt–lecithin micelle is around 25000–30000 according to calculations based on the K_{av} values obtained[19].

Lysolecithin forms clear solutions in water which from a physicochemical point of view are characterised as micellar. From light-scattering data the aggregate weight of the lecithin micelles has been calculated to be 95000, (ref. 22) and from surface tension data the critical micelle concentration was found to be 0.02–0.04 μmole/ml in water[23]. The effect of monovalent ions on the lowering of surface tension was found to be small. When 10 μmoles of lysolecithin in a 1-ml solution was applied to a gel column of 60 ml total volume, lysolecithin appeared in a peak with a K_{av} value between 0.05 and 0.10, corresponding to an aggregate weight of 80000–100000 which compares well with those reported from light-scattering measurements. Being a micellar solution in equilibrium with a molecularly dispersed phase, the lysolecithin micelles would be supposed to be more and more disaggregated when they pass down the column and meet an increasingly larger volume of buffer. The 1-ml sample applied will not, however, be diluted to more than 60 ml, *i.e.* the total volume of the column, and the critical micelle concentration for the lysolecithin is reported to be as low as 0.02–0.04 μmole/ml. If the 2 μmoles lysolecithin applied to the column were diluted over the total column the concentration would still be above the critical micelle concentration. In the present experiments the fractions after the peak contain a constant concentration of 0.003 μmole/ml lysolecithin, which could be expected to be the critical micelle concentration for lysolecithin under the conditions of our experiments. In an attempt to study the behaviour of molecularly dispersed lysolecithin, 0.1 μmole lysolecithin was applied to a column of 60 ml volume. No micellar peak was found; on the other hand, the recovery of lysolecithin from the column was low indicating irreversible adsorption to the column. The value of critical micellar concentration calculated above may therefore be too low.

Lysolecithin formed mixed micelles with bile salt which were eluted with a K_{av} comparable to that of the mixed lecithin–bile salt micelles, indicating an aggregate weight of 15–25000. Also mixed lysolecithin–monoolein–bile salt micelles were formed with the same aggregate size.

The above experiments thus indicate that, when sodium taurodeoxycholate is present in the solution, lecithin and lysolecithin exists in mixed bile salt micelles with an aggregate size somewhat larger than that of the pure bile salt micelles but considerably smaller than the lysolecithin micelles and very much smaller than the liquid crystals of the lecithin dispersions.

When interpreting the results of the incubation experiments it must be taken into consideration that the conditions of the experiments differ from those *in vivo* in several important aspects. *In vivo* a continuous transport is taking place of absorbed material away from the mucosa *via* the lymph and portal blood. *In vivo* there is also a continuous transport of the intestinal content in the distal direction to meet new mucosa cells. *In vitro*, under the conditions of our experiments, we are working with a closed system in which an equilibrium is formed between medium and cells with little transport back to the medium of metabolites formed in the cells from absorbed

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material. It has earlier been demonstrated that the uptake of fatty acids by intestinal slices *in vitro* is a physical process that does not require energy[17]. The subsequent metabolism, mainly acylation, is, however, energy-dependent and rate-limiting[17]. The end products of fatty acid metabolism of the intestinal epithelial cell, the chylomicrons, do not leak out from the intestinal slice preparation to any larger extent. In the present experiments there is, however, in some cases a certain amount of fatty acid metabolites, especially triglycerides, in the incubation medium at the end of the incubation period.

As earlier demonstrated[17] oleic acid in bile salt solution is "taken up" by intestinal slices *in vitro*; *i.e.*, it is distributed in favour of the cells and is incorporated mainly into triglycerides. In the present experiments a certain amount, 4–6% of the oleic acid present in the incubated tissue was found incorporated into lecithin. This amount was not influenced to any considerable degree by the presence in the incubation medium of lysolecithin. The maximum amount of absorbed oleic acid found in this experiment in the lecithin of the intestinal tissue was 9%.

When intestinal slices were incubated with α-acyl-labeled lysolecithin 50–70% was present in the tissue at the end of the incubation. Of the activity in the tissues, 40–50% was found as lecithin. Considerable amounts of the α-acyl fatty acid of lysolecithin, however, was present in triglycerides of the tissue and in the free fatty acid fraction of the incubation medium at the end of the experiment. The total amount of active fatty acid in the medium and tissue still present in lecithin or lysolecithin at the end of the incubation period was 32–55%. The higher percentage of fatty acid activity present in the lecithin of the intestinal tissue after incubation, when α-acyl-lysolecithin was in the incubation medium, compared with free fatty acid would suggest that an appreciable amount of the α-acyl-lysolecithin is taken up intact by the intestinal slices and converted to lecithin.

To study further the uptake of lysolecithin by the intestinal slices, choline-labeled lecithin was prepared from the livers of rats fed labeled choline. The lecithin was isolated and converted to lysolecithin by the action of bee venom lecithinase A. Palmitic and stearic acids are the dominant fatty acids of α-lysolecithin prepared from rat liver lecithin[28]. The uptake of the choline-labeled lysolecithin was of the same order as that of the acyl-labeled lysolecithin; of the lysolecithin taken up, 57–86% was found as lecithin. After incubation, 40–50% of the activity was present in non-lipid form indicating a splitting of the fatty acyl–ester bond in these experiments to about the same degree as with α-^{3}H-acyl label. This amount was only 13–17% when using inverted sacs, indicating that the lysolecithinase activity of the medium comes from ruptured mucosal cells.

Addition of bile salt to the incubation medium has no significant effect on the uptake of lysolecithin from the incubation medium; however, there seemed to be less triglyceride and more lecithin formed in the tissue in the absence of bile salt in the medium.

The tissue preparations show an effective conversion of absorbed lysolecithin to lecithin. Two different pathways have been described for the conversion of lysolecithin to lecithin.

Lysolecithin + acyl-CoA → lecithin + CoA (1) (ref. 24)

2 lysolecithin → lecithin + glycerylphosphorylcholine (2) (ref. 25)

If pathway (1) were a major pathway for the formation of lecithin in our experiments, more active fatty acid than choline from the lysolecithin would be incorporated into lecithin. The amount of labeled lecithin formed, however, was about the same in the incubations with choline-labeled lysolecithin as with α-acyl labeled lysolecithin. The results therefore indicate that the glycerylphosphorylcholine formed from lysolecithin is a product of a lysolecithinase reaction, as a rather large fraction of the labeled fatty acid originally present in the α position of lysolecithin is found in triglyceride in the tissue. The lecithin formed thus arises by acylation of lysolecithin according to pathway (1). It is known that lecithin can be formed by mucosal cells by the Kennedy pathway[29]. The incorporation of labeled fatty acid into lecithin however was considerably less in the incubations with labeled free fatty acids than with [α-*acyl*-^{3}H]lysolecithin and no activity was incorporated into lecithin in incubations with [*glyceryl*-^{3}H]phosphorylcholine. The main part of the labeled lecithin in our experiments therefore cannot be formed by active split products entering the Kennedy pathway.

In the experiments with lecithin in the incubation medium the uptake into the tissue is varying but generally appreciably lower than for lysolecithin. This is especially the case in the absence of bile salt in the medium and when the uptake was studied with choline-labeled lecithin. The largest uptake was generally found for β-acyl labeled lecithin and less for α,β-diacyl-labeled lecithin. When acyl label was used, a considerable amount of activity in the medium was found as free fatty acids and in the tissue as triglycerides. Considerable amounts of lecithin were also found in the incubation medium at the end of the experiments. It can therefore be assumed that most of the uptake was in the form of fatty acids split off from lecithin by the action of lecithinase A. The results of these experiments give no difinite evidence for uptake of intact lecithin whether bile salt was present or not.

Differing lecithinase activity in the medium could well explain the varying results with acyl-labeled lecithin. Even in the experiments in which the slices had been taken from animals, the bile ducts of which had been canulated 24 h prior to the experiments, the medium contained appreciable lecithinase activity.

In two incubations with [*choline-Me*-^{3}H]lecithin, 3–4% of the activity without and 9–10% with bile salt were obtained in the slices. The distribution of the activity in the tissue was the same as when lysolecithin labeled in the same way was used. The small differences obtained with and without bile salt in the medium could be explained by a slightly higher lecithinase activity in the presence of bile salt. Such a stimulation was found when lecithin was incubated in the supernatant from homogenized slices.

The present experiments thus show that lysolecithin *in vitro* is to a large extent taken up intact by intestinal slices and is acylated to lecithin. No definite evidence was found for the uptake of intact lecithin with or without bile salt.

Considering the fact that lyso compounds are the predominant species of glycero-phospholipids found in intestinal contents[4] *in vivo*, there is reason to believe that the lyso compounds are absorbed from the intestinal tract as such. The quantitative aspects of lysolecithin absorption have to await further studies with the intact animal.

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EXHIBIT 7

LECITHIN

	Usual Level %	Max. Level %	No. Companies Reporting
Soft Candy	0.18	0.32	26
Baked Goods	0.12	0.5	25
Fats and Oils	0.19	0.39	17
Confections and Frostings	0.34	0.40	9
Gravies	0.18	0.19	8
Meat Products	0.04	0.12	6
Imitation Dairy Products	1.17	1.89	4
Sweet Sauces	0.25	.66	4
Milk Products	1.33	1.33	3

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THE EFFECT OF PHOSPHOLIPID INGESTION UPON THE GAS EXCHANGE IN MAN[1]

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During the course of some researches to determine whether or not sprue patients can tolerate lecithin better than neutral fat, 60 grams of mixed soya bean phospholipids were fed to normal subjects by stomach tube and the respiratory quotient, urinary phosphate and nitrogen, blood sugar, serum inorganic phosphate and phospholipid determined. The surprising observation was made that the R.Q., under the conditions of the experiment, quickly dropped to a minimum in a half-hour and then rose to a maximum as high as unity in two to four hours. To determine to what extent this phenomenon was due to the phosphate, to the fatty acid, or to the phospholipid as a molecular unit, equivalent amounts of disodium glycerophosphate, of olive oil, and of mixtures of disodium glycerophosphate and olive oil were fed to subjects and the same determinations made.

LITERATURE. The effect upon the R.Q. of feeding lecithin or its non-lipid constituents has received but little attention.

Gregg (1932) obtained no change in R.Q. after feeding lecithin to dogs. Yet Gregg as well as Kovaliova (1912) obtained increases in R.Q. after injecting that phospholipid. This rise in R.Q. might be related to the observations of Izar and Constantino (1929) and of Jost (1931). The former reported a significant rise in blood sugar after the injection of lecithin and the latter a rise in the production of sugar when cephalin was added to the blood of a perfused liver.

Page and Young (1932) and Yriant (1931), working with phlorhizined and depancreatized dogs respectively, could not show this production of sugar from phospholipid.

Kovaliova (1912) and McCann and Hannon (1923) obtained a rapid lowering in R.Q. after the injection and ingestion respectively of glycerophosphates. Ablin (1925, 1926, 1929) and Schmutzer (1928) showed that when phosphate is fed with carbohydrate the rise in R.Q. is delayed.

[1] A preliminary report of this paper was presented before the American Society of Biological Chemists, April 1, 1938, at Baltimore, Maryland.

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The effect of fat feeding upon the gas exchange has received more attention, especially from Murlin and his students. Until 1933 Murlin believed, as did Lusk and many others, that all R.Q. values of less than 0.700 were due to errors in their determination or of some other effect not the direct result of metabolism. However, at this time work was done in his laboratory (Hawley et al., 1932, 1933) which showed quite conclusively that pigs and humans with a high tolerance for fat will give R.Q. values lower than 0.700 after ingesting fat and that the R.Q. will rise 0.04 to 0.06 unit above the basal level about seven hours after the fat meal. Later Murlin (1936) assumed that in R.Q. values below 0.707 all the non-protein CO_2 is from fat combustion and that the extra O_2 absorbed is concerned in partial oxidation of fat. He was not convinced, however, that the fat had been converted to carbohydrate, but pointed out that the fatty acids may be oxidized along the chain to some other product.

In most studies on R.Q. after the ingestion of fats no observations were made before one and a half or two hours after the test meal. As will be shown in the present work, however, the R.Q. is almost at its lowest level after one-half hour when small amounts of fat are fed. Gregg obtained such an effect shortly after feeding fat to rats (1931) and considered it as due to a transient acidosis and ketonuria. A similar observation was made by Wilder, Boothby and Beeler (1923) who were studying the metabolism of a diabetic. These authors made no effort to explain such an early drop.

Methods.[3] The respiratory quotient was determined by collecting the exhaled air for five minutes in a Douglas bag and analyzing it in a Haldane apparatus. Blood and urinary phosphates were determined by the method of Fiske and Subbarow (1925), phospholipid by the method of Boyd (1931), urinary nitrogen by the Kjeldahl method, blood sugar by Benedict's method (1931), and blood CO_2 content by the Van Slyke volumetric apparatus.

Experimental. In our earlier experments the phospholipid, as obtained from the Glidden Co.,[4] contained about 30 per cent of oil. This was removed by dissolving in petroleum ether and precipitating with acetone, the procedure being carried out several times. Later the Glidden Co. sent the material fat-free. The major portion of soya bean phospholipid is cephalin, the rest mainly lecithin. (Nottbohm and Mayer, 1932.)

In preparation for the experiments 60 to 65 grams of the purified preparation were emulsified in five volumes of water. The subjects were allowed no food or tobacco after 6:30 the evening preceding the experiment. At

[3] The gas analyses and urinary nitrogen determinations were made by Miss Hanes Clement.

[4] The soya bean phospholipids used in this study were generously supplied by The Glidden Company, of Chicago, Illinois.

TABLE 1

Metabolic and biochemical effects of the ingestion of 60 grams of phospholipid

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM PHOSPHOLIPID	MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS
		CO_2	O_2	R.Q.			
	hrs.						
J. B. A.	Basal	7.02	9.75	0.720	165	3.8	17
	½	7.24	10.15	0.714			
	1	7.49	9.55	0.785	187	4.2	13
	2	7.72	9.10	0.849	213	4.9	27
	3	7.96	8.35	0.954	195	6.3	63
	4	7.52	9.02	0.834	168		63
	5	9.44	10.85	0.870	161		32
	6	9.64	11.55	0.835		5.4	11
P. E.	Basal	6.00	7.41	0.810	165	4.2	40
	½	5.13	6.98	0.735			55
	1	6.39	8.31	0.770	165	4.3	70
	2	6.49	8.25	0.787	180	4.8	84
	3	6.47	8.03	0.806	187	4.8	85
	4	7.34	8.74	0.840	124	5.1	73
	5	6.59	8.51	0.774			73
E. D.	Basal	9.79	12.30	0.796		4.21	20
	½	9.44	12.50	0.755		4.13	10
	1	9.24	12.15	0.784		4.35	3
	2	9.51	11.70	0.814		4.50	35
	3	9.24	12.10	0.764		5.06	22
	4	10.44	13.40	0.780		4.71	17
	5	9.01	11.85	0.760		4.60	71
T. W. O.	Basal	9.39	12.73	0.738			
	½	8.86	11.58	0.765			
	1	8.52	11.26	0.756			
	2	8.84	10.76	0.822			
	3	9.19	10.45	0.880			
	4	9.44	11.45	0.824			
	5	8.77	11.02	0.795			
L. J.	Basal	3.95	5.59	0.707	158	4.17	20
	½	5.25	7.41	0.709		4.51	27
	1	5.14	6.97	0.738	159	5.08	31
	2	5.69	7.40	0.770	200	5.40	103
	3	5.32	7.12	0.747	183	5.95	111
	4	5.64	7.35	0.767	170	6.12	190
L. M.	Basal	5.70	7.55	0.736			
	1	5.84	8.14	0.718			
	2	6.60	8.07	0.818			
	3	6.06	7.89	0.777			
	4	6.36	8.47	0.751			

TABLE 2

Metabolic and biochemical effects of the ingestion of 60 grams of phospholipid (incomplete)

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM PHOSPHO-LIPID	MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS
		CO_2	O_2	R.Q.			
	hrs.						
C. R. D.*	Basal	Overbreathed			161		
	2	12.08	14.74	0.820	172		
	3	11.93	12.84	0.930	183		
	4	13.43	12.74	1.055	194		
	5	10.23	11.64	0.880	153		
	6	8.43	11.84	0.712	161		
J. F.	Basal	Overbreathed					18
	½	7.70	9.52	0.810	185	3.28	22
	1	8.73	10.08	0.866	205	4.20	25
	2	7.88	9.22	0.855		4.80	47
	3	8.00	9.50	0.843	213	5.00	87
	4	8.18	8.83	0.927	179	5.20	95
	5	7.68	8.47	0.907	179	5.50	112
	6	7.72	9.97	0.774			99
R. L. E.	Basal	Overbreathed			223	3.00	15
	½	7.07	7.70	0.920		3.12	17
	2	6.82	7.77	0.878	216	4.20	21
	3	7.63	7.41	1.040	223	4.75	37
	4	6.51	5.95	1.094	245	5.60	38
	5	6.43	5.78	1.113	245	5.80	55
	6	7.55	8.12	0.930	238	5.92	47
C. D.	Basal	Overbreathed			176		13.1
	½	9.06	11.06	0.818			9.28
	1	8.48	10.28	0.826	176		22.7
	2	8.93	9.74	0.917	176		57.8
	3	9.54	10.19	0.935	227		61.1
	4	8.23	9.39	0.878	193		64.0
	5	8.05	10.13	0.795	224		60.0
R. T.	Basal	Faulty analysis					
	½	6.79	8.75	0.777			
	1	7.73	9.75	0.793			
	2	7.95	9.70	0.820			
	3	7.25	8.60	0.843			
	4	8.62	9.58	0.900			
	5	8.26	10.37	0.795			

* This subject received 100 grams.

5:30 or 6:00 o'clock on the morning of the experiment they were asked to void as completely as possible and were given a glass of water. At about 7:30 they were wheeled on a chair or stretcher to the room in which the experiments were conducted. After a short rest the basal R.Q. was determined. In spite of the precaution of taking the subjects into the room the preceding day and training them in breathing into the bag, several over-breathed for the basal test. After the air had been collected for the basal R.Q. blood was collected for the determination of blood sugar, serum

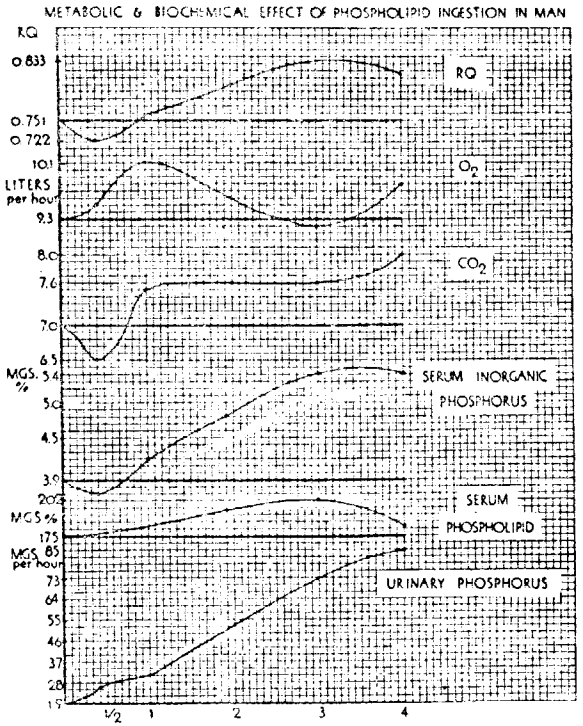

Chart 1

inorganic phosphate, and serum fats, and, in a few cases, blood was collected under oil for CO_2 content. After the blood was drawn the patient voided as completely as possible. Inorganic phosphate and total nitrogen were determined on the urine obtained.

The test meal was then given, the phospholipid being passed by stomach tube. In only one case did the subject become nauseated. This was C. R. D. who, because of his size, was given 100 grams of phospholipid. After that experience all subjects were given 60 grams with no untoward

effect. Experiments were continued from 4 to 6 hours, the air, blood, and urine collections being made in that order.

The results of the phospholipid experiments are given in tables 1 and 2 and chart 1. The blood sugar also was determined in many cases, but it did not change significantly. The R.Q. and gas values in the chart are only for those experiments in which trustworthy determinations were made in every period. Table 2, however, shows that in those experiments which were not complete there was a similar rise in R.Q.

TABLE 3

Metabolic and biological effects of the ingestion of 16 grams of disodium glycerophosphate

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITER PER HOUR)			MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS
		CO_2	O_2	R.Q.		
	hrs.					
I. W. B.	Basal			0.765		
	½			0.689		
	1			0.716		
	2			0.775		
	3			0.779		
	4			0.762		
J. P.	Basal	9.74	10.21	0.954	2.98	23.1
	½	7.09	9.98	0.710	3.35	24.4
	1	7.59	10.00	0.759	3.35	47.2
	2	8.57	10.33	0.830	3.57	60.0
	3	7.35	9.53	0.772	3.16	71.0
	4	7.45	9.93	0.750	3.16	75.0
C. B.	Basal	8.71	10.96	0.785	4.63	7.7
	½	7.58	9.63	0.796	4.63	10.9
	1¼	7.55	10.12	0.746	5.40	26.2
	2	7.92	10.62	0.745	5.71	37.4
	3	8.18	10.34	0.754	5.71	40.0
	4¾	8.34	10.34	0.769		43.0

Since the results of the phospholipid experiments were so different from those reported in the literature for fat, it was thought that the effects might be due to the phosphate. Accordingly, three subjects were given 16 grams of disodium glycerophosphate. In the first experiment only the total R.Q. was determined, but the last two patients were studied as completely as after phospholipid feeding. The results of these experiments are given in table 3.

Three subjects were given a mixture of 45 grams of olive oil with 16 grams of disodium glycerophosphate to determine if the effects of the

phospholipid were due to the molecule as such. The results are tabulated in table 4.

Finally, the effects of 45 grams of olive oil alone were determined on three subjects. The results are summarized in table 5.

DISCUSSION. The mean values of all the determinations are compared in chart 2. In order to have a basis for comparison all basal values were reduced to zero and the unit changes plotted. The R.Q. changes appear to show no interrelationship, those after phospholipid appearing to be

TABLE 4

Metabolic and biochemical effects of the ingestion of 45 grams of olive oil and 16 grams of disodium glycerophosphate

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS	MGM. PER CENT SERUM PHOSPHOLIPID
		CO_2	O_2	R.Q.			
	hrs.						
J. L.	Basal	10.44	12.07	0.866	3.75	42	198
	½	8.60	11.70	0.735	4.22	36	191
	1	9.35	10.35	0.904	5.00	53	176
	2	9.60	10.75	0.893	5.40	66	187
	3	9.73	11.62	0.837	5.26	68	187
	4	10.26	11.62	0.890	5.08	59	194
E. D.	Basal	8.76	11.20	0.782	4.16	12	175
	½	7.88	11.48	0.686	4.21	27	
	1	10.05	13.75	0.732	4.40	17	175
	2	9.29	12.37	0.751	4.95	30	187
	3	9.32	12.27	0.780	5.15	32	187
	4	10.16	12.45	0.816	5.15	69	180
	5	9.60	12.77	0.751	5.00	115	187
C. D.	Basal	8.07	9.15	0.882	4.30		194
	½	8.61	10.66	0.808	4.55	13.4	
	1	8.18	10.06	0.805	4.55	13.4	194
	2	8.11	9.78	0.830	5.13	28	194
	3	8.13	9.58	0.848	5.72	61	194
	4	8.39	10.51	0.798	5.72	60	194

quite different from those after its constituents. However, there is a similarity in the oxygen and carbon dioxide changes after oil plus phosphate to those after phospholipid and a dissimilarity of these changes to those after phosphate or oil alone. The changes in serum and urine inorganic phosphorus make it quite evident that the absorption of fat increases absorption of phosphate and that this effect is greatest after phospholipid. Verzar and Laszt (1934) have presented evidence indicating that glycerol phosphate increases the absorption of fat, although Irwin,

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Weber and Steenbock (1936) could not confirm it. An increase of 25 per cent in serum phospholipid after the ingestion of 60 grams of phospholipid, but none after equivalent amounts of olive oil or oil plus phosphate, presents the possibility that some of the complex compound may have been absorbed unchanged.

There are several possible explanations of the high R.Q. values after phospholipid ingestion. One of these is that it is an acidosis effect. However, the elevated R.Q. corresponds to a decrease in oxygen consumption

TABLE 5

Metabolic and biochemical effects of the ingestion of 45 grams of olive oil

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS	MGM. PER CENT SERUM PHOSPHOLIPID
		CO_2	O_2	R.Q.			
	hrs.						
B. L.	Basal	8.15	11.55	0.706	3.64	28	225
	½	8.13	11.68	0.696	3.46	17	233
	1	8.19	11.03	0.743	3.33	16	215
	2	8.35	10.80	0.773	3.68	19	222
	3	8.23	11.21	0.735	3.64	26	229
	4	8.83	11.85	0.745	3.59	20	
R. B.	Basal	6.76	7.67	0.882	4.21	47	
	½	4.92	6.07	0.811	4.04	42	
	2	7.09	9.37	0.757	4.12	36	
	3	8.18	9.66	0.846	4.45	39	
	4	7.26	8.77	0.827	4.45	47	
E. D.	Basal	9.79	12.30	0.796	4.21	20	187
	½	9.44	12.50	0.755	4.13	9.7	
	1	9.24	12.15	0.784	4.35	12	187
	2	9.51	11.70	0.814	4.50	15	191
	3	9.24	12.10	0.764	5.06	18	191
	4	10.44	13.40	0.780	4.71	36	202
	5	9.01	11.85	0.760	4.60	44	202

after an early increase, and not to a blowing off of carbon dioxide. Furthermore, the changes in the gas exchange after phosphate plus oil resemble closely the changes after phospholipid but are very different from the changes after either of these alone. It is thus unlikely that the effect is one of acidosis.

A second possibility is that feeding phospholipid stimulates carbohydrate metabolism. However, it hardly seems reasonable that the ingestion of phospholipid should depress fat metabolism and stimulate the mobilization and combustion of glycogen stores.

It is possible that the fatty acids of the ingested phospholipid undergo a partial oxidation in the intestinal mucosa during absorption. This would account for the observed increase in oxygen absorption. These partially oxidized fatty acids may then be carried to the tissues, where the oxidation is completed, causing a reduction in the amount of oxygen absorbed.

The possibility that fats are oxidized in a stepwise manner has been proposed from time to time. Very recently Werthessen (1937), by feeding

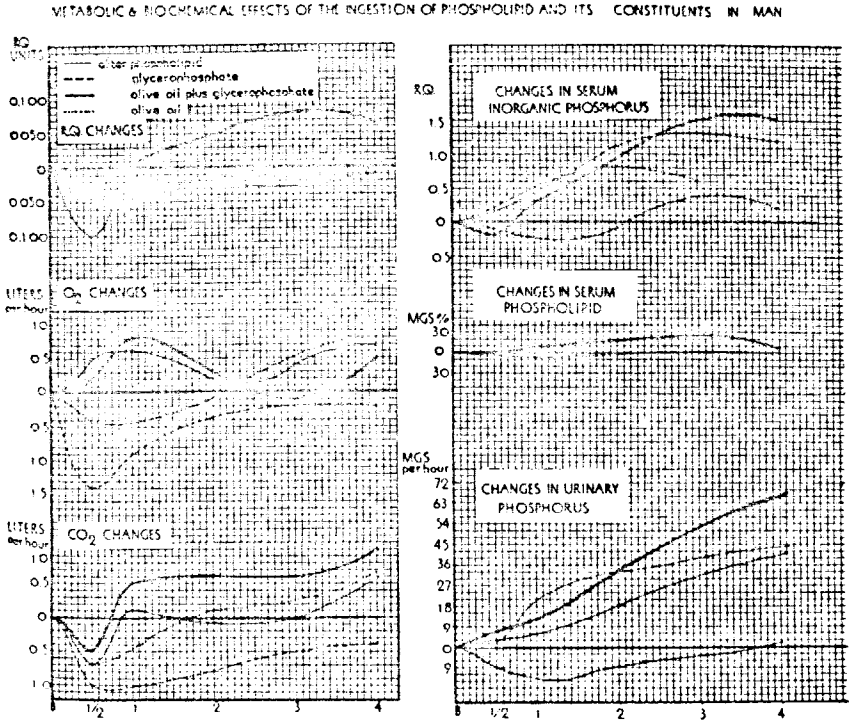

Chart 2

rats at a certain time of the day and no other, obtained R.Q. values ranging from 0.27 to 1.70. He explained these results by a chain reaction system in which the fats are first converted to a complex carbohydrate and then to a simple one of the glucose type.

It is probable that only a portion of the ingested fat goes through these stages during absorption, the major part being absorbed as fat and stored. Any condition, therefore, which would cause a larger part of the absorbed fat to be oxidized in the manner described would show evidence of the

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stepwise oxidation in the gas exchange. The presence of phosphate would appear to be such a condition since phosphate and fat apparently aid each other reciprocally in absorption, and the gas exchange after feeding the two together presents a different picture from that after either alone. Unpublished work in this laboratory shows that there is a lowering of blood and urinary inorganic phosphate during the absorption of fat, which is further evidence that phosphate is a factor in the absorption and metabolism of the lipids.

SUMMARY AND CONCLUSIONS

1. It was found that after feeding 60 grams of soya bean phospholipids to humans the non-protein R.Q. fell during the first half-hour and then rose, reaching a maximum considerably above the basal value in three to four hours. It was shown that this was mainly an oxygen effect.

2. That this effect was not entirely one of the phospholipid molecule as such was shown by similar effects upon the oxygen absorption and carbon dioxide elimination of a mixture of olive oil and disodium glycerophosphate equivalent in amount to the phospholipid. In the latter condition, however, the R.Q. did not rise above the basal level.

3. That the effect is not due to the phosphate or oil alone is shown by the very different results after feeding these substances separately.

4. The increase in serum inorganic phosphate was greatest after phospholipid feeding, slightly less after oil plus phosphate, and much less after phosphate alone.

5. The elimination of urinary phosphate was greatest after phospholipid, less but more rapid after phosphate, and somewhat still less and slower after oil plus phosphate.

6. An increase of 25 mgm. per cent in serum phospholipid after phospholipid feeding was not found after feeding equivalent amounts of olive oil or oil plus phosphate.

7. The significance of these findings is discussed.

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The action of some synthetic lysolecithins and lecithins on erythrocytes and lipid bilayers

GOTTFRIED AND RAPPORT[1] have investigated the hemolytic action of a series of lyso phosphoglycerides. The nature of the linkage of the hydrocarbon chain did not appreciably affect lytic activity, but unsaturation in the parafinnic chain of lysolecithin was found to reduce the hemolytic action. In order to obtain additional information on the relevance of chemical structure to hemolytic activity we assayed a number of synthetic lysolecithins and lecithins containing fatty acid constituents of different chain lengths. In addition, the action of some of these compounds on lipid bilayers was studied.

The following lecithins were prepared by acylation of glycero-3-phosphoryl choline[2,3]: 1,2-dimyristoyl-glycero-3-phosphoryl choline ((dimyristoyl)lecithin), 1,2-didecanoyl-glycero-3-phosphoryl choline ((didecanoyl)lecithin) and 1,2-diheptanoyl-glycero-3-phosphoryl choline ((diheptanoyl)lecithin). The synthesis of 1-oleoyl-2-butyryl-glycero-3-phosphoryl choline ((1-oleoyl-2-butyryl)lecithin) was as described by BIRD *et al.*[4]. Hydrolysis of lecithins with snake venom phospholipase A furnished 1-stearoyl-glycero-3-phosphoryl choline ((stearoyl)lysolecithin), 1-oleoyl-glycero-3-phosphoryl choline ((oleoyl)lysolecithin), 1-myristoyl-glycero-3-phosphoryl choline ((myristoyl)lysolecithin) and 1-decanoyl glycero-3-phosphoryl choline ((decanoyl)-lysolecithin). The purity of the phospholipids was confirmed by thin-layer and paper chromatography. Known amounts of these compounds were emulsified in 0.9% saline by vigorous shaking. Beef erythrocytes were washed 3 times with 0.9% saline and the cells were suspended in 0.9% saline to give suspensions with a 50% hematocrit. The lysis test was carried out by adding a small volume of the lysin solution to 5 ml of 0.9% saline in the cuvette of a Vitatron spectrophotometer, and the transmission was adjusted at 100%. Subsequently 50 μl of the erythrocyte suspension was added and the alteration of transmission at 625 mμ was recorded as a function of time. The time required to give 50% hemolyses at various concentrations of lytic agent is represented in order to facilitate a comparison with the bilayer experiments. Lipid bilayers were generated from a solution of total lipid from beef erythrocytes (1% lipid in decane) in an apparatus essentially the same as that described by THOMPSON[5].

Measurements were made of the average survival time of these films at various concentrations of lytic agent injected into one of the compartments.

Lysis of erythrocytes was affected by (stearoyl)lysolecithin, (myristoyl)lysolecithin and (oleoyl)lysolecithin in that order (Fig. 1A). However, in comparison to these compounds (decanoyl)lysolecithin revealed a lytic action of a much lower order, 2.0 μmoles/ml being necessary to give an onset of lysis under the conditions used. The experiments indicate that the hemolytic activity of lysolecithin is highly dependent on the chain length and further experiments with lysolecithin analogs with fatty acid constituents between C_{10} and C_{14} can give us further information about this relationship. In agreement with the observations of GOTTFRIED AND RAPPORT[1] (oleoyl)-lysolecithin was found to be less active as compared with (stearoyl)lysolecithin; moreover, the time required to give 50% hemolysis was increased for the unsaturated

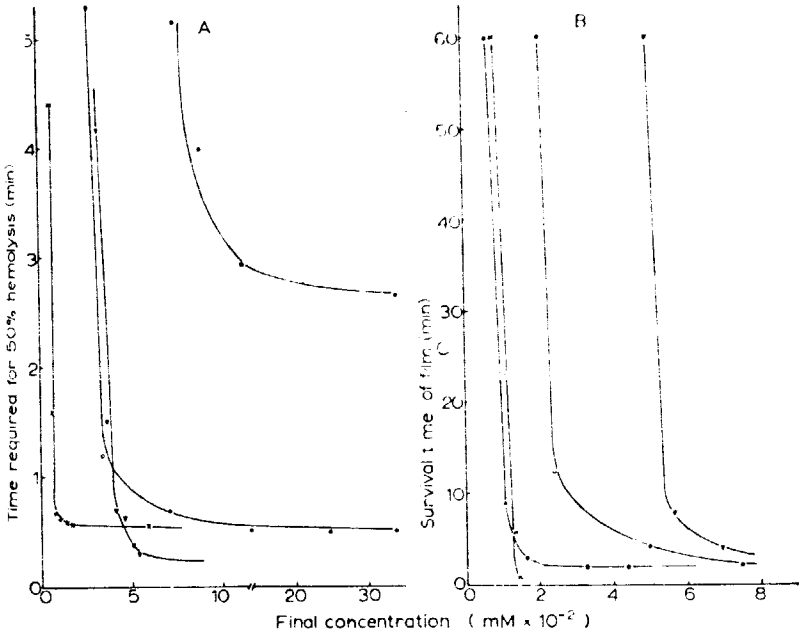

Fig. 1. Lytic action on beef erythrocytes (A) and bilayers of lipids from beef erythrocytes (B) of (stearoyl)lysolecithin (×——×), (myristoyl)lysolecithin (▼——▼), (oleoyl)lysolecithin (●——●) and (didecanoyl)lecithin (○——○).

compound. Not only monoacyl phosphoglycerides, but also certain diacyl derivatives can exert hemolytic action as shown by the results with (didecanoyl)lecithin (Fig. 1A) the action of which equals that of (myristoyl)lysolecithin. It is interesting to note that the activity of this lecithin is of a much higher magnitude than that of its so-called lyso derivative. As regards the other lecithins tested, (diheptanoyl)lecithin required a minimum concentration of 2.2 μmoles/ml to cause lysis whereas (dimyristoyl)-lecithin caused no lysis at concentrations up to 1.5 μmoles/ml. These observations have to be extended to other lecithins with chain length between C_7 and C_{14} in order to assess whether (didecanoyl)lecithin is the most lytic lecithin of the saturated series. A mixed acid lecithin, *viz.* (1-oleoyl-2-butyryl)lecithin, was found to be weakly hemolytic (lysis at 0.6 μmole/ml). This compound has been found to give micelles of a type similar to lysolecithin–lecithin mixtures[6,7]. Further studies on the size and shape of micelles of various synthetic lysolecithins and lecithins are in progress. The lipid bilayers were not lysed by (dimyristoyl)lecithin at the relatively high concentrations which were sublytic to beef erythrocytes. On the other hand, the lysolecithins containing stearic, myristic and oleic acid as well as the lytic (didecanoyl)lecithin rapidly disrupted these lipid bilayers at concentrations which are of the same order of magnitude as those found for the lysis of erythrocytes (Fig. 1B). These results support the view that the hemolysis of erythrocytes by lysolecithins has to be attributed largely to an interaction with the lipid constituents of the red cells. On the other hand, the lipid bilayers showed some interesting differences in susceptibility as compared with erythrocytes, *viz.* in the membrane (stearoyl)- and (oleoyl)lysolecithins gave a nearly

similar behaviour whereas these compounds exhibited different lytic activities for erythrocytes. It appears that further studies on the action of lysolecithins and lecithins of different chemical constitution on erythrocytes and lipid bilayers may give useful information about the mechanism of lysis. In this context it is of interest to note that VAN ZUTPHEN AND VAN DEENEN[9] observed that, in membranes generated from lecithin–lysolecithin mixtures, a most significant decrease in the electric resistance of the lipid bilayer occurred at a critical concentration of a given lysolecithin.

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The effect of emulsification of a milk-substitute diet by mechanical homogenization and by the addition of soya-bean lecithin on plasma lipid and vitamin A levels and on the growth rate of the newborn calf

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Although experiments on the effect of inclusion of vegetable or animal fats in synthetic milks (Wiese, Johnson, Mitchell & Nevens, 1947; Lofgreen, 1950; Kastelic, Bentley & Phillips, 1950) or in milk substitutes based on dried skim-milk powder (Gullickson, Fountaine & Fitch, 1942) have been made over a number of years, it is only comparatively recently that milk substitutes consisting basically of skim milk and added fat have been used commercially. This development has come from an increasing interest in veal production which, especially on the continent of Europe, takes the form of intensive production that requires a specialized diet of high energy content, which at the moment can most economically be achieved by utilizing fats other than butterfat. However, the type of added fat and also the method by which it is incorporated into the skim milk is of great importance owing to the sensitivity of the calf to dietary changes, particularly during the 1st month of life.

Gullickson *et al.* (1942) obtained very poor results with homogenized-milk diets containing 3·5% of either cottonseed, soya-bean or maize oil, but the animal fats, lard and tallow, gave moderately good results and coconut oil gave an intermediate response. These workers reported loss of hair, first from the face, ears and neck and later from the cannons of the rear legs, of calves given the maize, cottonseed or soya-bean oil. Bate, Espe & Cannon (1946), however, associated similar hair losses with unhomogenized diets, whereas de Man (1951) found that diets containing unhydrogenated soya-bean oil caused hair loss and development of eczema due to excretion of oil through the skin, whereas hydrogenated soya-bean oil had much less effect. Even when homogenized, unhydrogenated soya-bean oil has been found to cause a poor rate of growth, rough hair coats and a high incidence of scouring and mortality (Jacobson & Cannon, 1947; Wiese *et al.* 1947; Jacobson, Cannon & Thomas, 1949; Murley, Jacobson, Wise & Allen, 1949; Barker, Wise & Jacobson, 1952; Adams, Gullickson, Gander & Sautter, 1959), but diets containing hydrogenated soya-bean oil have given results nearly as good as those containing butter oil (Murley *et al.* 1949).

Raven & Robinson (1958, 1959, 1960) have studied the nutritive value of palm oil and palm-kernel oil for the calf, and have found the digestibility of both hydrogenated

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and unhydrogenated forms to be lower than that of butterfat. The hydrogenated palm oil appeared to have a lower protein-sparing effect than the unhydrogenated oil; however, these oils were not compared directly in the same experiment.

The finding of Gullickson *et al.* (1942) that lard and tallow appeared to be well utilized by the calf has been confirmed by many workers (Wiese *et al.* 1947; Blaxter & Wood, 1951; Johnson, Hopper & Gardner, 1953; Hopper, Gardner & Johnson, 1954; Ritchey, Hopper, Gardner & Johnson, 1956; Thomas & Okamoto, 1956; Bell, 1958; Larsen, 1958; Comberg & Göllnitz, 1959; Kliesch & Horst, 1959; Kesler, Wilson & Moore, 1960), but Jacobson, Brown & Ratcliff (1959) found that the incidence of scouring was higher with a diet containing lard than with whole milk.

Lard and tallow appear to be of equal value, but Brüggemann & Barth (1959) found that the digestibility of lard was superior to that of beef suet, although both were well digested from 15 to 60 days of age. Hydrogenated herring oil has been used in Norway (Homb, 1960), but the calves did not grow as well as those given butterfat, though both groups were much superior to those given coconut fat, which caused severe scouring.

Nearly all the diets containing added fats so far mentioned have been mechanically homogenized since there is general agreement that for efficient utilization some form of treatment is necessary to reduce the size of the fat globules. Kastelic *et al.* (1950) found that, when a synthetic milk containing 1% cottonseed oil was emulsified with soya-bean lecithin in a Waring Blendor, a calf given the diet scoured within 2 days. However, when a mixture of water, cottonseed oil and lecithin that had been homogenized at a pressure of 1800 lb/in^2 until the oil globules were 2μ or less in diameter was given to this calf (now 12 days of age), the diarrhoea stopped within 36 h. On the basis of this slender evidence it has been assumed that a fat globule size of less than 2μ should be aimed at in milk substitutes for calves.

There is little doubt that the size of fat globule is of importance, for the smaller the globule the greater is the rate of digestion, and with a large proportion of small globules more protein may be adsorbed on the globule surfaces. The diameter of fat globule in cow's milk varies from 0·1 to 10μ with an average of $3–4\mu$ (dalla Torre, 1953; Ling, Kon & Porter, 1961), and cow's milk that contains large fat globules also tends to form a tougher curd than one containing small globules (Clements, 1949). Recently, it has been suggested that rats given homogenized milk *ad lib.* gain more in live weight and utilize protein better than those fed on an untreated milk (Petrilli & Agnese, 1960). Moreover, it has been suggested that the small fat globules of butter and high-quality margarine can be absorbed intact, but other fats (olive oil and some margarines) have to be emulsified first and are passed through the intestinal wall mainly as fat degradation products and only partly as fine emulsions (Haubold, Heuer & Rohusinskyi, 1960).

The use of emulsifying agents as an alternative to homogenization has been studied for some years. Huff, Waugh & Wise (1951) observed a loss of hair in calves when glycerol monostearate was used as the emulsifying agent in a diet containing hydrogenated cottonseed oil, but when the diet was homogenized the characteristic alopecia

was absent; recently Hopkins, Warner & Loosli (1959) have shown that emulsification of coconut fat with crude soya-bean lecithin resulted in a very large increase in apparent digestibility of the fat, and mechanical homogenization of the diet had no additional beneficial effect. However, these calves were given antibiotics and the digestion trials were not made until the calves were 2–3 weeks of age. Increased digestibility of certain fats with increasing age of the calf has been observed by Cunningham & Loosli (1954), Lassiter, Duncan & Christie (1957) and Brüggemann & Barth (1959).

Hodgson & Murdock (1960) found that a milk substitute containing on a dry-matter basis 10% stabilized lard together with skim milk, whey and buttermilk caused significantly greater weight gains in calves when the lard was homogenized in the buttermilk than when the fat was just physically blended, but they do not mention whether an emulsifying agent was used. Both diets gave better results than a milk substitute containing no fat.

In many of our experiments we have used margarine in a milk substitute and have obtained apparent digestibilities of fat of up to 98% during the first 3 weeks of life (Shillam, 1960). However, the mixture had always been homogenized at a pressure of 200 atm. It was therefore of interest to know whether lecithin could be used as a substitute for mechanical homogenization and whether its inclusion in a diet had any beneficial effect other than that resulting from its role as an emulsifying agent. In earlier work (Aschaffenburg, Bartlett, Kon, Roy, Sears, Thompson, Ingram, Lovell & Wood, 1953) it was shown that the inclusion of lecithin in the first two feeds of life greatly increased the absorption of vitamin A, but this effect may have been particularly marked during this time when the intestine was permeable to the passage of whey proteins into the lacteals (Comline, Roberts & Titchen, 1951). However, it is known that lecithin increases the rate of absorption of lipids in rats and in man (Adlersberg & Sobotka, 1943; Augur, Rollman & Deuel, 1947; Tidwell, 1950).

The effect of soya-bean lecithin was therefore studied in three experiments. In the first, two forms of soya-bean lecithin, a crude and a pharmaceutical grade, were compared. They were included in a milk-substitute diet into which the margarine had been mechanically homogenized. In the second and third experiments, mechanical homogenization of margarine fat was compared with emulsification with lecithin.

METHODS

Plan of experiments

All three experiments were of randomized block design.

Expt 1. It was done in the early months of 1959 and consisted of three treatments in each of six blocks, four of Ayrshire bull calves and two of Shorthorn, as follows:

Treatment no.	Basal diet	Lecithin given
1	Reconstituted spray-dried skim milk containing 2% margarine incorporated by homogenization	None
2		Pharmaceutical grade
3		Crude grade

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Expt 2. It was done in the autumn and winter of 1959 and consisted of four treatments in each of twelve blocks, eight of Ayrshire bull calves and four of Shorthorn, as follows:

Treatment no.	Basal diet	Method of incorporating fat
4	Reconstituted spray-dried skim milk containing 2% margarine	Homogenization
5		Homogenization and emulsification with the pharmaceutical grade of lecithin
6		Emulsification with the pharmaceutical grade of lecithin
7		No emulsification or homogenization

Expt 3. This was done in the early summer of 1960 and consisted of two treatments in each of twelve blocks, ten of Ayrshire bull calves and two of Shorthorn, as follows:

Treatment no.	Basal diet	Method of incorporating fat
8	Reconstituted spray-dried skim milk, containing 2% margarine	Homogenization
9		Emulsification with the pharmaceutical grade of lecithin before reconstitution

Diets

Colostrum. Colostrum obtained within 24 h of calving from Shorthorn and Ayrshire cows was used; 1-pint samples were stored separately in waxed-paper cartons at $-25°$. Each calf was given initially 6 pints of colostrum consisting of 1 pint from each of six different batches. Calves within each block received the same blend of colostrum, but there were differences in the blend between the blocks.

Milk-substitute diets. The basal diet consisted of margarine fat and of reconstituted spray-dried skim-milk powder that had been processed at a pre-heating temperature of 77° for 15 sec only and in which no apparent denaturation of the non-casein protein had occurred (Shillam, 1960; Shillam, Dawson & Roy, 1960).

The margarine contained no additional vitamins and consisted of approximately 39% groundnut oil, 24% palm oil and 37% coconut oil, of which a proportion was in the form of hardened oil. The lecithin content of the margarine was about 0·003% but approximately 0·18% of emulsifiers ('monoglyceride based on fully hardened groundnut oil') was included during manufacture.

For the diets used in treatment 1 of Expt 1, treatment 4 of Expt 2 and treatment 8 of Expt 3, a milk of 20% fat content was prepared by homogenizing 2 lb non-vitaminized margarine into a reconstituted mixture of 0·8 lb spray-dried skim-milk powder in 7·2 lb water. It was made once or twice weekly, a maximum of 50 lb being prepared at any one time, and stored at 4° until required. A small single-action type of homogenizer was used, working at a pressure of 200 atm; a stirrer was fitted close to the inlet so that the fluid entered the machine in a well-mixed condition.

Each 10 lb batch of the basal diet was prepared by reconstituting 0·9 lb of skim-milk powder with 8·1 lb of water to which was added 1 lb of the milk of 20% fat content.

Lecithin. In Expt 1 two forms of soya-bean lecithin (American Lecithin Company Inc.) were used: one, Alcolec S, a feeding grade in a carrier of soya-bean oil with a

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phosphatide content of 62–65 %, and the other, Alcolec granules, a pharmaceutical grade made by repeated washing of the Alcolec S with acetone to remove the soya-bean oil and containing 95 % of phosphatides (J. Eichberg, 1959, personal communication). Before homogenization 47·8 g of Alcolec granules were dissolved in each 2 lb of margarine used in the preparation of the diet given to calves on treatment 2 and 69·8 g of Alcolec S in each 2 lb of margarine used in the diets given to calves on treatment 3. Thus the final concentration of lecithin was 0·1 %.

In Expt 2, only the pharmaceutical grade of lecithin was used at a concentration of 0·2 %. For treatment 5, 95·6 g of Alcolec granules were added to each 2 lb margarine before homogenization. For treatment 7, each 10 lb batch of milk of 20 % fat content was prepared by stirring 2 lb of the melted fat into 0·8 lb skim-milk powder dissolved in 7·2 lb of water; for treatment 6, 95·6 g of Alcolec granules were first added to the melted fat.

In Expt 3, melted fat containing the pharmaceutical grade of lecithin at a concentration of 2 % was poured slowly into the skim-milk powder as it was stirred in a Hobart mixer. Stirring was continued for 5–10 min until the mixture became cream-like in consistency; it was then cooled to 4°. This mixture was diluted with water to give a diet containing 2 % margarine and 0·2 % lecithin.

Each calf received 3500 i.u. vitamin A in the form of halibut-liver oil concentrate and 700 i.u. synthetic vitamin D_3 daily, both vitamins being dissolved in arachis oil. The diets were given in quantities that had been found adequate for a growth rate of 1 lb/day when whole milk of 3·3 % fat content was used (Roy, Shillam, Hawkins & Lang, 1958).

Calves

The calves were reared for 3 weeks; collection and management were as in earlier experiments (Aschaffenburg, Bartlett, Kon, Terry, Thompson, Walker, Briggs, Cotchin & Lovell, 1949). If a calf scoured, the quantity of milk was reduced to that calculated to maintain body-weight (Roy *et al.* 1958). When the consistency of the faeces returned to normal, the volume of milk was increased to the normal daily allowance. This practice was repeated when scouring recurred.

The mean dry-matter content of the faeces was calculated for each calf during the experimental period from daily subjective grading of the consistency of the faeces on a scale increasing from 0 (severe diarrhoea) to 7 (very firm faeces). The mean values obtained from dry-matter determinations on several hundred samples of faeces were:

Grade	0	1	2	3	4	5	6	7
Dry matter (%)	5	7	10	14	22	26	28	32

Jugular blood was drawn from all the calves in Expts 1 and 2 before their first feed and about 4 h after their morning feed on the 4th, 12th and 21st days of age. Total plasma lipids were extracted by the method of Jacobson, Zaletel & Allen (1953). After weighing the fat, vitamin A was chromatographically separated (Ganguly, Kon & Thompson, 1947).

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RESULTS

Expt 1. The results are given in Tables 1 and 2. The addition of the pharmaceutical grade of lecithin to the homogenized diet had no effect on the performance of the calves. However, calves given lecithin in a soya-bean oil carrier showed a significantly greater incidence of a high rectal temperature (> 102·8 °F) and, after adjustment for differences between treatment groups in mean milk consumption and incidence of scouring, a significant increase in live-weight gain/day. The relevant partial regression coefficients with their standard errors were:

	General mean	Partial regression coefficient with its standard error
Live-weight gain/day (lb)	0·690	—
Total milk consumption (pints)	158·71	0·0081 ± 0·0027*
No. of days on which scouring occurred (values transformed $\sqrt{(x+\frac{1}{2})}$)	1·158	−0·1924 ± 0·0519**

* Significant at $0{\cdot}01 < P < 0{\cdot}05$.
** Significant at $0{\cdot}001 < P < 0{\cdot}01$.

As shown in Table 2, the addition of lecithin had no apparent effect on plasma lipids during the first 12 days of life; the levels rose more or less uniformly throughout the experimental period except for calves given the crude grade, which showed a fall between the 12th and 21st days. With all treatments the plasma content of vitamin A rose rapidly during the first 4 days of life, but this rise was followed by a slight fall to the 12th day. The plasma vitamin A levels of the calves given the diet containing the crude lecithin continued to fall to the 21st day whereas those of the calves on the other two diets showed a slight rise; the difference was significant.

Expt 2. The results are given in Tables 3 and 4. Diets prepared without homogenization resulted in very poor live-weight gains; inclusion of lecithin in the homogenized diet had no significant effect on live-weight gain, but there was some suggestion of an effect with the unhomogenized diet. After adjustment of live-weight gain for differences between treatment groups in mean birth weight and milk consumption the effect of lecithin was still not significant. The relevant partial regression coefficients were:

	General mean	Partial regression coefficient with its standard error
Live-weight gain/day (lb)	0·423	—
Birth weight (lb)	76·26	−0·0121 ± 0·0035**
Total milk consumption (pints)	144·22	+0·0096 ± 0·0034**

** Significant at $0{\cdot}001 < P < 0{\cdot}01$.

No difference between treatments in the incidence of scouring was apparent, but the estimated mean dry-matter content of the faeces was increased both by homogenization and by emulsification with lecithin.

Loss of hair on the head and legs was apparent to a marked extent only in calves given the unhomogenized and unemulsified fat; unaltered fat was plainly visible in the faeces of these calves, especially during the 1st fortnight of life.

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Table 1. *Expt* 1. *Comparison of the performance (mean values with their standard errors) of calves given homogenized milk-substitute diets containing two different preparations of soya-bean lecithin*

	Treatment no. and details			
	1 No lecithin	2 Granular lecithin (95 % phosphatides)	3 Lecithin in soya-bean oil carrier (65 % phosphatides)	Significance of difference between treatments
of calves	6	6	6	—
weight (lb)	84·0 ± 3·51	82·7 ± 3·51	75·0 ± 3·51	—
milk consumption (pints)	164·5 ± 4·42	156·5 ± 4·42	155·1 ± 4·42	—
weight gain/day (lb)	0·77 ± 0·073	0·57 ± 0·073	0·74 ± 0·073	—
of days on which calves scoured	0 (range 0–1)	2 (range 0–6)	1 (range 0–3)	—
matter in faeces (%)	23·0 ± 1·0	20·0 ± 1·0	22·0 ± 1·0	—
of days on which calves had a rectal temperature (> 102·8 °F)	0 (range 0–2)	1 (range 0–2)	3 (range 0–7)	3 > 1*
sted live-weight gain/day (lb)†	0·67 ± 0·041	0·64 ± 0·039	0·76 ± 0·037	3 > 1+2*‡

Significant at $0{\cdot}01 < P < 0{\cdot}05$.
Adjusted for differences between treatment groups in mean milk consumption and incidence of scouring.
Overall mean value for treatments 1 and 2.

Table 2. *Expt* 1. *Comparison of levels of blood plasma lipids and vitamin A (mean values with their standard errors) of calves given homogenized milk-substitute diets containing two different preparations of soya-bean lecithin*

	Treatment no. and details				
	1 No lecithin	2 Granular lecithin (95 % phosphatides)	3 Lecithin in soya-bean oil carrier (65 % phosphatides)	Pooled standard error of means	Significance of difference between treatments
of calves	6	6	6		
d plasma vitamin A 100 ml):					
At birth	3·8 ± 0·67	3·5 ± 0·67	3·5 ± 0·76†		—
At 4 days	8·4	7·3	8·3	± 1·21	—
At 12 days	7·6	6·1	7·3	± 0·70	—
At 21 days	9·3	8·6	6·9	± 1·01	—
Increase, 4th–21st day	+ 1·0	+ 1·4	− 1·4	± 0·70	3 < 1*, 3 < 2*
d plasma lipids 100 ml):					
At birth	68	101	107	± 13·0	1 < 2+3*‡
At 4 days	182	157	196	± 22·5	—
At 12 days	277	245	284	± 36·4	—
At 21 days	324	329	280	± 40·2	—

* Significant at $0{\cdot}01 < P < 0{\cdot}05$.
† One sample not analysed. Missing value calculated by the missing-plot technique of Yates (1933).
‡ Overall mean value for treatments 2 and 3.

Table 3. *Expt 2. Comparison of the performance (mean values with their standard errors) of calves given milk-substitute diets containing margarine fat incorporated by different methods*

	Treatment no. and details				
	4 Homogenization	5 Lecithin + homogenization	6 Lecithin	7 No lecithin or homogenization	Significance of difference between treatments
No. of calves	12	12	12	12	
Birth weight (lb)	75·5 ±2·99	72·5 ±2·99	76·2 ±2·99	80·7 ±2·99	—
Total milk consumption (pints)	146·6 ±3·16	142·8 ±3·16	140·3 ±3·16	147·1 ±3·16	—
Live-weight gain/day (lb)	0·58 ± 0·054	0·57 ± 0·054	0·32 ± 0·054	0·22 ± 0·054	5 > 6**, 5 > 7***, 4 > 6**, 4 > 7***, 4+5 > 6+7***†
No. of days on which calves scoured	3 (range 0–6)	2 (range 0–7)	2 (range 0–10)	3 (range 0–9)	—
Dry matter in faeces (%)	19 ± 0·5	20 ± 0·5	19 ± 0·5	17 ± 0·5	4+5 > 6+7*†, 5+6 > 4+7*†, 4 > 7*, 5 > 7**, 6 > 7*
No. of days on which calves had a high rectal temperature (> 102·8 °F)	1 (range 0–7)	2 (range 0–10)	2 (range 0–6)	1 (range 0–4)	—
Adjusted live-weight gain/day (lb)‡	0·55 ± 0·048	0·54 ± 0·049	0·36 ± 0·048	0·24 ± 0·049	4 > 7***, 5 > 7***, 4 > 6*, 5 > 6*

* Significant at $0·01 < P < 0·05$.
** Significant at $0·001 < P < 0·01$.
*** Significant at $P < 0·001$.

† Two treatment nos. joined by a plus sign indicate the mean value of those treatments.
‡ Adjusted for differences between treatment groups in mean birth weight and milk consumption.

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Table 4. *Expt 2. Comparison of levels of blood plasma lipids and vitamin A (mean values with their standard errors) of calves given milk-substitute diets containing margarine fat incorporated by different methods*

	Treatment no. and details				Pooled standard error of means	Significance of difference between treatments
	4 Homogenization	5 Lecithin and homogenization	6 Lecithin	7 No lecithin or homogenization		
No. of calves	12	12	12	12		
Blood plasma vitamin A (μg/100 ml):						
At birth	3·3	5·0	4·1	3·7	±0·50	—
At 4 days	6·0	5·9	5·2	6·4	±0·47	—
At 12 days	5·2	5·0	5·2	4·4	±0·39	—
At 21 days	5·8±0·49	5·7±0·54†	5·7±0·49	4·6±0·49	—	—
Increase, birth–4 days	+2·7	+0·9	+1·1	+2·7	±0·44	4+7 > 5+6***§
Adjusted increase, birth–4 days‡	+2·4±0·39	+1·3±0·40	+1·1±0·38	+2·6±0·38	—	{4+7 > 5+6**§, 4 > 6*, 7 > 5*, 7 > 6**
Regression coefficient of increase in plasma vitamin A from birth to 4 days on birth value, −0·4454±0·1316**						
Blood plasma lipids (mg/100 ml):						
At birth	168	145	146	183	±26·7	—
At 4 days	195	222	180	148	±21·8	4+5 > 6+7*§
At 12 days	242	286	188	185	±29·2	4+5 > 6+7*§
At 21 days	315±21·5	265±23·6†	304±21·5	247±21·5	—	4+6 > 5+7*§
Increase, birth–4 days	+27	+77	+34	−35	±33·7	—
Adjusted increase, birth–4 days‡	+35±22·2	+63±22·2	+21±22·2	−13±22·4	—	5 > 7*
Regression coefficient of increase in plasma lipids from birth to 4 days on birth value, −0·9618±0·1441***						

* Significant at $0·01 < P < 0·05$.
** Significant at $0·001 < P < 0·01$.
*** Significant at $P < 0·001$.
† One sample not analysed. Missing value calculated by the missing-plot technique of Yates (1933).
‡ Adjusted for differences between treatments in mean birth value.
§ Two treatment nos. joined by a plus sign indicate the mean value of those treatments.

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Microscopic examination of the size of fat globule in the diets showed that the mean diameter of the homogenized globule was about 3–4 μ, and that the globules in the product emulsified with lecithin were probably of the mean size of 10–20 μ with some as large as 50 μ.

As is apparent from Table 4, lecithin at this level of 0·2 % significantly depressed the increase in plasma vitamin A levels between birth and the 4th day of life. After adjustment of the increase for differences in the value of vitamin A at birth by means of the regression coefficient given in Table 4, the effect was still significant. The interaction of lecithin and homogenization was significant when the increase in vitamin A levels from the 4th to the 12th day of life was analysed. However, the decline in plasma vitamin A between the 4th and 12th days was significantly greater for calves given the unemulsified fat than for those on the other three treatments.

Plasma lipid values on the 4th and 12th days of life were significantly increased by homogenization, whereas lecithin was ineffective. The increase in content of plasma lipids from birth to 4 days of age, after adjustment for the value at birth, was much greater for calves given the diet that was both emulsified and homogenized than for calves given unemulsified fat. However, the plasma lipid value for the 21st day indicated that homogenization and emulsification with lecithin were having an antagonistic effect. Thus the increase in levels of plasma lipids of calves given the diet that was both homogenized and emulsified was similar to that obtained for those given a diet containing unemulsified fat.

Table 5. *Expt* 3. *Comparison of the performance (mean values with their standard errors) of calves given milk-substitute diets containing margarine fat incorporated either by homogenization into reconstituted skim-milk powder or together with lecithin directly into the dry powder*

	Treatment no. and details		Significance of difference between treatments
	8 Homogenization	9 Lecithin	
No. of calves	12	11	
Birth weight (lb)	81·3 ± 2·74	83·7 ± 2·98†	—
Total milk consumption (pints)	149·7 ± 1·91	152·2 ± 2·08†	—
Live-weight gain/day (lb)	0·49 ± 0·055	0·35 ± 0·060†	—
No. of days on which calves scoured	3 (range 1–7)	3 (range 0–5)	—
No. of days on which calves had a high rectal temperature (> 102·8 °F)	1 (range 0–5)	2 (range 0–6)	—
Adjusted live-weight gain/day (lb)‡	0·48 ± 0·033	0·36 ± 0·036†	8 > 9*

* Significant at $0{\cdot}01 < P < 0{\cdot}05$.
† Missing value calculated by the missing-plot technique of Yates (1933).
‡ Adjusted for differences between treatment groups in mean birth weight and milk consumption.

Expt 3. The results are given in Table 5. One calf given the diet in which the fat was emulsified with lecithin died from an *Escherichia coli* localized intestinal infection. As in Expt 2, the mean growth rate of the calves given the homogenized diet, after adjustment for differences between treatment groups in mean birth weight and milk consumption, was greater than that of calves given the diet in which the fat was

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emulsified with lecithin. The partial regression coefficients used for the adjustment were:

	General mean	Partial regression coefficient with its standard error
Live-weight gain/day (lb)	0·419	—
Birth weight (lb)	82·49	−0·0196 ± 0·0043**
Total milk consumption (pints)	150·91	0·0142 ± 0·0061*

* Significant at $0{\cdot}01 < P < 0{\cdot}05$.
** Significant at $0{\cdot}001 < P < 0{\cdot}01$.

There was no difference between the two treatments in the incidence of scouring.

DISCUSSION

From the results of Expt 1, it is apparent that the addition of lecithin to an homogenized diet was of little beneficial effect, a finding in agreement with that of Bell (1958). The slightly higher adjusted live-weight gain of calves given the diet containing crude, but not the pharmaceutical grade of, lecithin was unexpected and may have been due to some effect other than emulsification. Hopkins *et al.* (1959) considered such a possibility when they found that crude lecithin markedly improved the digestibility of tallow and coconut fat. Moreover, Allen, Jacobson, Ward & Zaletel (1956) found that in calves given crude soya-bean oil, the proportion of linoleic acid in the plasma total fatty acids was much higher than in those given a diet containing milk fat, hydrogenated soya-bean oil or lard. However, Spalton (1953) has pointed out that highly purified lecithin is not as good an emulsifier as the less refined grades.

The results of Expt 2 show that under our conditions mechanical homogenization is a more effective method of incorporating fat into a reconstituted skim-milk diet than emulsification of the fat with soya-bean lecithin. The use of lecithin, however, had a slight but not significant beneficial effect on growth rate.

In spite of the often accepted opinion that unemulsified fat causes scouring, particularly in very young calves, the only evidence of digestive disorders in this experiment was a slightly lowered dry-matter content of the faeces in the calves given unemulsified fat. Lecithin significantly increased the dry-matter content of the faeces and also largely prevented the loss of hair from the body that has been associated with unemulsified-fat diets. From our observations, the loss of hair was caused by direct contact with the unemulsified-fat diet and also with the faeces passed by calves receiving this diet rather than being the result of a systemic effect.

Contrary to the results of Expt 1, in which only the crude lecithin caused a depression in vitamin A level from the 12th to the 21st day, in Expt 2 the purified lecithin significantly depressed the increase in vitamin A between birth and 4 days of age. However, the concentration of lecithin in the diets in Expt 2 was twice that in Expt 1. The only other effect of treatment on plasma vitamin A level was a tendency for it to be lower between the 12th and 21st days in the calves given the diet containing unemulsified fat.

The level of plasma lipids during the first 12 days of life was increased in calves given an homogenized diet, whereas inclusion of lecithin had no effect. After the

12th day of age, it would appear that homogenization was of less importance, since the blood lipid content of the calves given fat emulsified with lecithin was very similar at 21 days of age to that of the calves given an homogenized diet.

The results for live-weight gain in Expt 3 merely confirmed those obtained in Expt 2, namely that a diet containing homogenized fat gave a better live-weight gain than one in which the fat was emulsified with lecithin. Moreover, intimately mixing the melted fat and lecithin with the dry skim-milk powder, as practised in the manufacture of some proprietary milk substitutes, gave results very similar to those obtained by mixing the melted fat and lecithin into reconstituted skim-milk powder.

It is thus clear that under our conditions we have been unable to confirm the finding of the Cornell workers (Hopkins *et al.* 1959) that mechanical homogenization gives no additional beneficial effect over emulsification of a fat with lecithin. However, their results were obtained with a diet containing coconut fat which had been emulsified with a crude lecithin, and with calves older than those used by us. It must be borne in mind also that a particular emulsifying agent may be more satisfactory with one type of fat than with another.

To be of maximum value, milk-substitute diets should be suitable for calves from 4 days of age. Some reduction in digestibility and utilization of fat during early life may have to be accepted for economic reasons, provided such diets do not predispose calves to scouring and a high death rate. However, in our experience, diets that are slightly inferior nutritionally to whole milk usually cause a very high incidence of mortality once other predisposing factors, such as a build-up of 'infection' (Roy, Palmer, Shillam, Ingram & Wood, 1955), begin to take effect. Thus, when a milk substitute contains a relatively large amount of fat, it would seem advisable to use an homogenized product during at least the first 2 weeks of life rather than one in which the fat had been emulsified with lecithin.

SUMMARY

1. Ninety newborn bull calves were used in three experiments to study the effect of inclusion of soya-bean lecithin in a milk-substitute diet, based on margarine and spray-dried skim-milk powder, during the first 3 weeks of life.

2. In the first experiment, the effect of addition to a homogenized diet of a pharmaceutical grade of lecithin and of lecithin in a soya-bean oil carrier was studied. The latter product gave a greater live-weight gain, after adjustment for differences between treatment groups in mean milk consumption and incidence of scouring, and a greater incidence of a high rectal temperature ($> 102{\cdot}8$ °F) and lowered plasma vitamin A and blood lipid levels between the 12th and 21st days of life.

3. In the second experiment, mechanical homogenization of the fat, emulsification of the fat with soya-bean lecithin and addition of the fat without any emulsification were compared. Homogenization significantly increased growth rate and the dry-matter content of the faeces. Emulsification with lecithin had a slight, but not significant, beneficial effect on live-weight gain and significantly increased the dry-matter content of the faeces.

The changes in plasma content of vitamin A between birth and 4 days of age and in the plasma lipid content up to 12 days of age were increased by homogenization. By 21 days of age, lipid levels of calves given a fat emulsified with lecithin were as high as those of calves given the homogenized diet.

4. In the third experiment, incorporation of margarine and lecithin into the dried skim-milk powder gave a significantly smaller live-weight gain, after adjustment for differences between treatment groups in mean birth weight and milk consumption, than a diet in which the fat had been incorporated by homogenization.

5. For liquid diets based on dried skim milk and containing 2% of fat, it is suggested that mechanical homogenization should be used in preference to emulsification with lecithin, especially during the first 2 weeks of life.

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THE LIVER LIPIDS IN NORMAL DOGS ON DIFFERENT TYPES OF FAT, WITH AND WITHOUT ADDED LECITHIN

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In the course of a study of the effect of lecithin on the liver lipids of depancreatized dogs, data were also obtained with the same animals before pancreatectomy, in order to observe the comparative effect of the diets used in the depancreatized and the normal group.

EXPERIMENTAL

Diets—The basic diet consisted of:

Raw lean beef	150 gm.
or Meat powder (Valentine)	35 "
Skim milk powder (Borden)	30 "
Cracker meal	100 "
Dried brewers' yeast	10 "
Salt mixture (Cowgill (1))	3 "
Bone ash	4 "
Viosterol	2 drops

Raw lean beef was fed to fifteen of the animals, meat powder to eighteen. Dogs weighing more than 15 kilos were given in addition 30 gm. of sucrose daily. Fat was added to the diet in one of three forms: (*a*) 20 ml. of cod liver oil (Mead Johnson), (*b*) 5 ml. of a 0.3 per cent solution of carotene in oil (S.M.A.) and 15 ml. of Mazola, or (*c*) 30 gm. of Crisco. Cod liver oil and carotene were used because we were also studying their effect on the vitamin A and carotene contents of the livers of these animals. Of the seventeen dogs receiving cod liver oil, six were given in addition a supplement of 10 gm. of crude egg yolk lecithin (John Carle and Son). Of the sixteen animals receiving the carotene and Mazola mixture,

seven were fed the lecithin supplement. None of the five animals fed Crisco was given lecithin. As analyzed in this laboratory, the lecithin preparation contained 94.2 per cent of material soluble in alcohol-ether, of which 72.8 per cent was lecithin. The Crisco diet was deficient in vitamin A; otherwise all the dogs received adequate amounts of vitamins A, D, and the B complex. In each group animals were maintained on the diets for varying periods. The minimal periods ranged from 10 to 48 days, the maximal from 216 to 406 days. The animals were kept in separate metabolism cages and fed daily at 4 p.m.

Sampling of Liver—All samples were removed under ether anesthesia in the postabsorptive state, 22 hours after the preceding meal. In taking samples for analysis, we avoided the peripheral margin of the liver samples.

In several instances, dogs (Nos. 134, 140, 142, 143, 161) subjected to repeated sampling of the liver have been included with the other normal animals. It will be noted that the lipid values observed in these cases fall within the range of the others. It is therefore concluded that sampling *per se* caused no enduring aberrations in the liver lipids.

Extraction of Lipids from Liver—The liver tissue (5 to 10 gm.) removed at operation was weighed and ground immediately with a known amount of sand. The material was transferred quantitatively with 150 ml. of alcohol-ether (3:1) to a tared flask and refluxed for 1 hour at about 55°. The extract, cooled to room temperature, was then filtered through a weighed fat-free filter paper into a 500 ml. volumetric flask. The extraction was repeated for 1 hour periods with alcohol-ether and finally with ethyl ether only. The combined extracts were made up to 500 ml. and mixed thoroughly.

Petroleum Ether Extract—An aliquot (200 to 300 ml.) of the alcohol-ether extract was concentrated at 40–50° under reduced pressure to near dryness, acidified with a drop of 2 N HCl, and immediately taken up in 100 ml. of petroleum ether. After standing overnight (2), the petroleum ether extract was filtered into a 200 ml. volumetric flask and made up to volume. Aliquot portions of this extract were taken for the determination of total lipid, lipoid phosphorus, unsaponifiable material, total fatty acids, and the iodine number of the total fatty acids.

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Total Lipids—These were determined by weighing the residue of an aliquot of the petroleum ether extract that had been evaporated to dryness.

Lipoid Phosphorus—The method of Fiske and Subbarow (3, 4) was employed on suitable aliquots of the petroleum ether extract.

Unsaponifiable Material—A third aliquot of the petroleum ether extract was saponified for 2 hours in alcoholic solution under nitrogen with 1 or 2 ml. of 50 per cent potassium hydroxide solution. The unsaponifiable fraction was extracted with petroleum ether, evaporated to dryness, and weighed.

Total Fatty Acids—After acidification of the saponified residue with HCl, the total fatty acids were extracted with petroleum ether. Aliquots were taken for weight and iodine number. The micromodification by Yasuda (5) of the Rosenmund and Kuhnhenn method was used for iodine number determination.

Free and Total Cholesterol—Aliquots of the original alcohol-ether extract were used for the determination of free and total cholesterol by a modification of the microgravimetric digitonin method described by Man and Peters (4) for serum cholesterol. For free cholesterol, 2 ml. of a 0.5 per cent solution of digitonin in 80 per cent alcohol were added to the alcohol-ether aliquot, and evaporated to dryness. The aliquot for total cholesterol was saponified with 0.1 ml. of saturated potassium hydroxide, redissolved in alcohol, acidified with HCl, and, after the addition of 2 ml. of 0.5 per cent digitonin, evaporated to dryness. The cholesterol digitonide, both free and total, was filtered into Jena fritted glass funnels, porosity G4, of 10 ml. capacity and weighing less than 8 gm. These were weighed on a microbalance. It was noted that a precipitate was sometimes formed, particularly in the total cholesterol determinations, which from its appearance and behavior was obviously not cholesterol digitonide and which when weighed with the digitonide gave erroneous results. Therefore the cholesterol digitonide was removed from the filter, after being weighed, by dissolving it in the funnel in several portions of boiling methyl alcohol, as recommended by Boyd (6). This was sucked through and the filter was washed with methyl alcohol and ether, dried, and weighed again.

Values for total cholesterol obtained by this procedure agreed, within the limits of experimental error, in samples taken from the

original alcohol-ether extract, with aliquots of the unsaponifiable fraction. Recovery of known amounts of cholesterol added to tissue extracts varied between 96 and 103 per cent.

Water in Liver—The tared filter paper and its contents were placed in the extraction flask and the whole dried at 40° in a warm air oven for several days, and then weighed. To the weight of dry defatted liver so obtained was added the total lipid to give the value for total solids. The difference between the wet weight of the liver and the total solids was calculated as the water content of the liver.

From the experimental values obtained from the above procedures, the other lipid values were calculated. *Cholesterol* combined as *ester* was obtained by subtracting the free from the total cholesterol. *Phospholipid* and *phospholipid fatty acids* were computed from the petroleum ether-soluble phosphorus as stearyl-oleyl lecithin (7) (3.85 per cent P, 71 per cent fatty acids). The value for *"neutral fat"* was obtained by subtracting from the value of the total fatty acids, the phospholipid fatty acids plus the fatty acids in combination with cholesterol as esters, and then dividing by 0.95 to convert the residual fatty acids thus obtained to triglycerides.

All solvents employed in this investigation were freshly distilled. The ethyl alcohol gave no test for aldehydes, the ethyl ether was peroxide-free, and the petroleum ether (distilling below 50°) gave no detectable blank in the estimation of the iodine number.

DISCUSSION

Agreement of Duplicates—The average percentage deviations from the mean in duplicate analyses of the same liver extract were as follows: total lipid 1.3, unsaponifiable 1.8, total fatty acids 1.0, iodine number of total fatty acids 2.5, lipoid phosphorus 2.0, free cholesterol 2.2, total cholesterol 3.0.[1]

Estimation of the total lipid by summation of the phospholipid, neutral fat, and the unsaponifiable fractions leads to values which are, on the average, 10 per cent lower than those obtained directly

[1] At the request of the Editors the detailed data summarized in Tables I and II are not included in this paper. The detailed data will be referred by the authors to anyone who is interested.

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TABLE I

Mean Values and Estimates of Standard Deviation, s, for Lipid Values of Different Dietary Classes

All values, except the iodine numbers, are expressed in per cent of original tissue.

Supplement to basal diet	No. of samples	Total lipid		Unsaponifiable		Total fatty acids		Iodine No. of total fatty acids		Phospholipid		"Neutral fat"		Cholesterol					
														Total		Free		Ester	
		Mean	s	Mean	s	Mean	s	Mean	s	Mean	s	Mean	s	Mean	s	Mean	s	Mean	s
Cod liver oil	11	5.03	0.54	0.72	0.20	2.80	0.27	145	7.2	3.06	0.54	0.76	0.32	0.216	0.026	0.202	0.027	0.011	0.020
" " " and lecithin	6	4.67	0.25	0.51	0.13	2.58	0.29	147	5.5	2.88	0.16	0.67	0.22	0.234	0.030	0.192	0.023	0.042	0.024
Mazola and carotene	9	4.83	0.85	0.72	0.22	2.70	0.40	128	10.1	3.02	0.50	0.69	0.25	0.262	0.037	0.212	0.029	0.049	0.026
" carotene, lecithin	7	4.82	0.59	0.72	0.34	2.70	0.34	135	8.4	3.00	0.37	0.69	0.19	0.280	0.046	0.218	0.029	0.062	0.025
Crisco	5	4.41	0.66	0.56	0.16	2.58	0.32	129	10.2	3.01	0.20	0.59	0.29	0.255	0.011	0.206	0.007	0.049	0.017
Grand mean	38	4.81	0.62	0.67	0.19	2.69	0.32	137	11.5	3.01	0.41	0.70	0.25	0.255	0.034	0.207	0.026	0.049	0.022

TABLE II

An Analysis of Variance According to Fisher

	Total lipids	Unsaponifiable	Total fatty acids	Iodine No. of total fatty acids	Phospholipid	Cholesterol			"Neutral fat"	Water
						Free	Ester	Total		
No. of observations	38	38	37	35	37	36	36	36	36	37
" " subclasses	5	5	5	5	5	5	5	5	5	5
$\sigma_2:\sigma_1$	0.97	1.43	0.76	2.74	0.43	0.96	0.91	1.35	0.57	1.02
Critical range	1.635	1.635	1.637	1.640	1.637	1.638	1.638	1.638	1.638	1.637
	0.417	0.417	0.417	0.417	0.417	0.417	0.417	0.417	0.417	0.417

σ_1 and σ_2 are modified standard deviations indicating, respectively, variance *within* and *among* classes (Fisher (11)). The statistical procedure is discussed by Page *et al.* (12).

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by weighing the residue from an aliquot of the petroleum ether extract. A systematic check of the procedure failed to make apparent any reason for this deficiency. Similar discrepancies have been reported by others. In a study of serum lipids, Wilson and Hansen (8) found that the saponifiable fraction averaged 81 per cent of the total lipoid phosphorus, the unsaponifiable phospholipid ranging between 7 and 32 per cent of the total. After making allowance for this fraction, these authors found that, "The material unaccounted for averaged 3.8 per cent of the total lipid [as determined by the gravimetric method of Wilson and Hanner (9), in which there is no saponification], the sum of the three fractions being less than the total in every instance." Man and Gildea (10) were similarly able to recover only 82 per cent of the theoretical amount of fatty acids of a purified lecithin saponified with potassium hydroxide. These authors therefore add 18 per cent of the phospholipid fatty acids to their values for total fatty acids. Since our saponification procedure follows closely that of Man and Gildea, it is interesting to note that the application of their correction factor to our data causes the mean estimate of total lipids by summation to agree within 2 per cent with the directly determined value.

Statistical Analysis—The results of a statistical analysis of the detailed data are given in Tables I and II. The results shown in Table I suggest that, except for the variations in the iodine numbers of the total fatty acids, there is no significant difference in any lipid fraction among the various dietary classes. This conclusion is borne out, as shown in Table II, by the results of an analysis of variance according to Fisher (11), with the notation of Page *et al.* (12). In no case, except for the iodine numbers, does the critical ratio lie between $\sigma_2:\sigma_1$ and unity, indicating with a probability of 90 per cent that there is no greater variation among the five classes than would be expected in any one class.

Regrouping the data in other ways revealed no significant differences which might depend on whether the animals received meat powder or lean beef, or on the length of time that a given diet was fed. In the latter connection, it is of interest to note that ingestion of the lecithin supplement for extended periods of time had no apparent effect on the phospholipid content of the liver.

SUMMARY

The results of lipid analyses of the livers of thirty-three normal dogs by a microgravimetric technique are reported. When divided into five classes, according to the type of fat added to the basal diet, the total lipids and the individual lipid fractions, except for the iodine numbers of the total fatty acids, show no statistically significant differences among the classes.

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In Vitro and in Vivo Effects of Inositol Phosphatide (Lipositol) Fraction of Soybean on Serum Lipids and Lipoproteins[1]

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ABSTRACT

SACHS, BERNARD A., ETHEL DANIELSON, BERNARD BURACK AND LOUIS LEITER. *In vitro and in vivo effects of inositol phosphatide (lipositol) fraction of soybean on serum lipids and lipoproteins.* J. Appl. Physiol. 13(3): 381–384. 1958.—Previous *in vitro* studies have shown that the alcohol insoluble inositol phosphatide (lipositol) fraction of soybean phosphatide complex produces an increase in the electrophoretic migration velocity of serum lipoproteins, while the alcohol soluble lecithin fraction is without effect. Extension of these studies revealed that the increase in electrophoretic migration velocity of serum lipoproteins produced by addition of the lipositol fraction to serum was inhibited by the addition of protamine *in vitro*, but was not inhibited by heating to 56°C. Lipositol isolated from the alcohol-insoluble fraction was found to be the compound responsible for the observed effects. The *in vitro* effects on lipoproteins were confirmed *in vivo* by intravenous administration of the lipositol fraction to normal rabbits. These changes were not accompanied by an increase in clearing factor activity. In the normal rabbit the 'lipoprotein shift' is accompanied by a prompt and profound mobilization of neutral fat and cholesterol to the blood stream.

IN PREVIOUS *in vitro* studies (1) we noted that the alcohol-insoluble fraction of whole soybean phosphatide complex[2] (containing approximately $\frac{2}{3}$ lipositol and $\frac{1}{3}$ cephalin) produced an increase in the electrophoretic migration velocity of lipoproteins when incubated with normal or hyperlipemic human sera. The alcohol-soluble fraction (containing approximately $\frac{2}{3}$ lecithin and $\frac{1}{3}$ cephalin) had no effect. These studies were extended to further evaluate the observed effect *in vitro* and in the rabbit *in vivo*.

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[1] Supported in part by grants from Lipotropic Research Foundation, Eli Lilly and Co. and G. D. Searle and Co.

[2] The alcohol insoluble fraction of soybean phosphatide was kindly supplied by Mr. J. Eichberg of American Lecithin Co., Astoria, N. Y. Sterile emulsions of the fraction were generously prepared by Drs. Lloyd L. Ely and R. Pool of Don Baxter, Inc., Glendale, Calif.

MATERIALS AND METHODS

In Vitro Studies. Human sera were analyzed for protein and lipoprotein by paper electrophoresis (2) before and after treatment as follows: *a) Effect of heat.* Five or ten milligrams of the inositol phosphatide fraction was added to one milliliter of human serum and heated in constant temperature water bath for 1 hour at 56°C. *b) Effect of protamine.* To half of 10 duplicate 1-ml samples of normal or hyperlipemic sera containing 5 or 10 mg of inositol phosphatide fraction, 0.2 mg or 5 mg protamine sulfate as a 1% solution was added. Both the serum-phosphatide and the serum-phosphatide-protamine samples were incubated for 24 hours at 37°C. *c) Effect of inositol.* In a similar manner, 5 mg inositol was added to 1 ml normal serum and incubated at 37°C.

Isolation Studies. Lipositol was isolated by the method of Woolley (3), modified in that the starting material was the alcohol-insoluble fraction rather than the whole soybean phos-

phatide complex. Five milligrams of the isolated lipositol was added to one milliliter of human sera, incubated 24 hours at 37°C and then subjected to paper electrophoretic analysis, as described above.

In Vivo Studies. Fifty milliliters of a 5% emulsion of the lipositol fraction in 5% dextrose were administered intravenously to 21 white New Zealand rabbits. Arterial ear blood was collected prior to and at intervals during the first 4 hours and during the 2 weeks following the infusion. Control studies were done utilizing 50 ml of 5% dextrose in 4 rabbits. The separated sera were analyzed for total lipid, lipid phosphorus and cholesterol, proteins and lipoproteins (2). Clearing factor activity was determined by the method of Grossman (4) in three of the rabbits given the lipositol fraction intravenously.

RESULTS

In Vitro Studies. Increase in migration velocity of serum lipoproteins, as the result of addition of the lipositol fraction to human sera, was not inhibited by heating to 56°C for 1 hour, nor by *in vitro* addition of 0.2 mg protamine sulfate. However, 5 mg protamine sulfate did prevent the increase in the migration velocity produced by 5 mg of the lipositol fraction. Incubation of human sera with inositol did not produce the increase in migration velocity of the lipoproteins observed with the inositol phosphatide (lipositol) fraction.

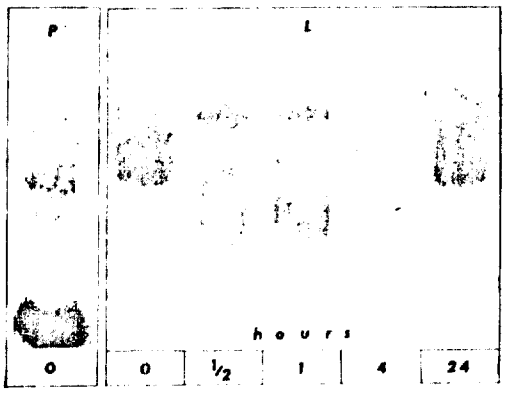

FIG. 1. Effect of intravenous administration of lipositol fraction emulsion on serum lipoproteins of the rabbit. *P* = proteins stained with naphthalene black 12B200, *L* = lipoproteins stained with oil red O.

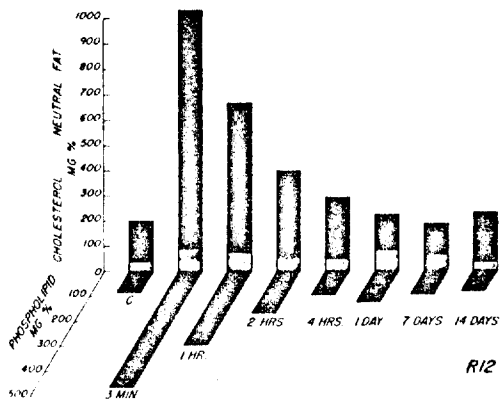

FIG. 2. Effect of lipositol fraction infusion on serum phospholipid (*oblique black bars*), cholesterol (*gray bars*) and neutral fat (*upright black bars*) of rabbit. *C* = initial values.

Isolation Studies. The purification technique of Woolley (3) for lipositol resulted in a compound containing 13.2% inositol and 2.8% phosphorus (as compared to Woolley's product, 15% inositol and 3.1% phosphorus). This compound, when incubated with human sera and analyzed electrophoretically, produced the 'lipoprotein shift' of the alcohol-insoluble fraction and the whole soybean phosphatide complex.

In Vivo Studies. Intravenous administration of an emulsion of the lipositol fraction to 21 rabbits produced the increase in migration velocity (previously noted *in vitro*) within 3 minutes. This lipoprotein shift was still present at the end of 4 hours. At the end of 24 hours, the lipoproteins had returned to their initial distribution (fig. 1). Dextrose-infused control rabbits showed no change in lipoproteins.

One hour after the lipositol fraction infusion, marked increases in serum lipids were noted and the average increments in 10 rabbits were as follows: phospholipid + 372%, cholesterol + 93% and neutral fat + 124%.

Associated with the rise in phospholipid due to phosphatide infusion there was within 3 minutes a marked increase in neutral fat and a definite, but less striking, rise in cholesterol (fig. 2). These values returned to normal by 4 hours and were slightly elevated after 24 hours. In 7 days, initial values for phospholipid and neutral fat were reached, but a mild elevation in serum cholesterol was still present

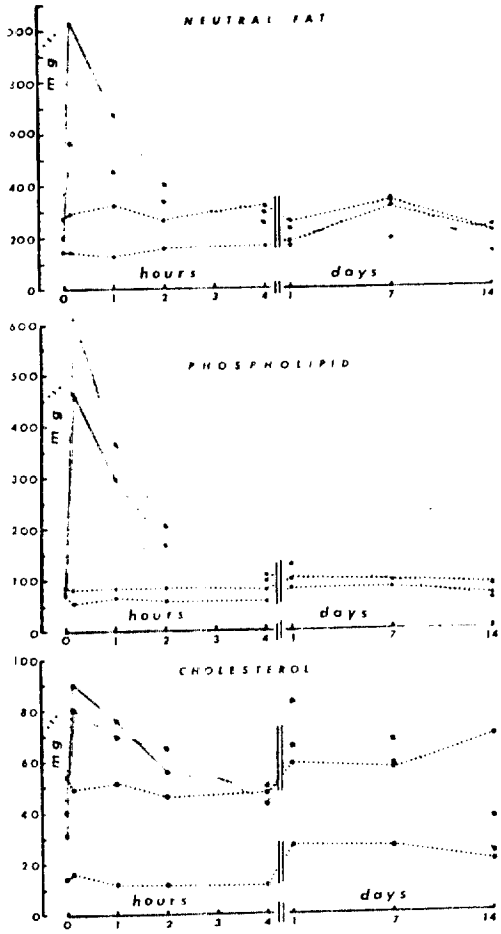

FIG. 3. Comparative effects of lipositol fraction infusions (*solid lines*) with dextrose infusions (*dotted lines*) on the serum lipid fractions of 4 rabbits.

In 14 days, the cholesterol levels returned to initial values.

The changes in phospholipid, neutral fat and cholesterol in rabbits, after lipositol fraction infusion, were compared with changes in rabbits after control infusions of 5% dextrose (fig. 3). There were no changes in phospholipid or neutral fat in the dextrose control animals. Similarly, the cholesterol values remained unchanged during the first 4 hours. After 24 hours, the dextrose-infused rabbits showed elevation in cholesterol levels similar to the phosphatide-infused animals.

No clearing factor activity was produced in rabbits given the lipositol fraction infusion.

DISCUSSION

In vitro studies suggest that the 'lipoprotein shift' is not produced by a heat-labile enzymatic reaction, for heating to 56° C did not inhibit this effect. Despite the fact that protamine did inhibit this *in vitro* reaction, failure to demonstrate a concomitant increase in free fatty acids or clearing factor activity suggests that the mechanism causing the increase in migration velocity of the serum lipoproteins differs from that produced by heparin (5).

We believe that lipositol is the compound responsible for the lipoprotein shift because our previous studies have shown that the alcohol-soluble fraction of soybean phosphatide which contains lecithin and cephalin does not produce the shift, because inositol alone is inactive and because lipositol actively produces the increase in migration velocity of the serum lipoproteins when isolated from the alcohol-insoluble fraction.

Lever and Waddell (6) demonstrated that the serum cholesterol of patients with idiopathic hyperlipemia and hypercholesteremic xanthomatosis falls following intravenous administration of an emulsion of 10% cottonseed oil and 1% of the whole soybean phosphatide. The effect is similar to that found in our cholesterol-fed rabbits. These authors later showed that the administration of the emulsion produced an increase in both electrophoretic mobility and clearing factor activity and that these effects could be produced by the phosphatide emulsifier when used alone (7). Because of the increase in clearing factor activity and because protamine reversed the effect, they postulated that heparin or heparin-like substance was liberated. In our experiments, in which the lipositol fraction instead of the whole phosphatide complex is used, we have found no increase in free fatty acids (1) and no increase in clearing factor activity, suggesting that another mechanism accounts for the observed effects.

Friedman and Byers (8, 9) have shown that a sustained infusion of whole phosphatide complex produces hypercholesterolemia in the hepatectomized or intact rat or rabbit. This suggests that cholesterol was brought to the blood from parts of the body other than the liver. This group has also demonstrated resolution of plaques from the aortas of previously

hypercholesteremic rabbits after 4 or 5 sustained infusions of phosphatide emulsion (10). Our studies demonstrate that a sustained phosphatide infusion is not essential for the production of hyperlipemia; a profound hyperlipemia was noted quickly following 10-minute infusions of the lipositol fraction to normal rabbits.

The authors express their thanks to Dr. J. Philip Savitsky for his helpful advice and criticism and to Dr. H. Seratng for performing the clearing factor determinations

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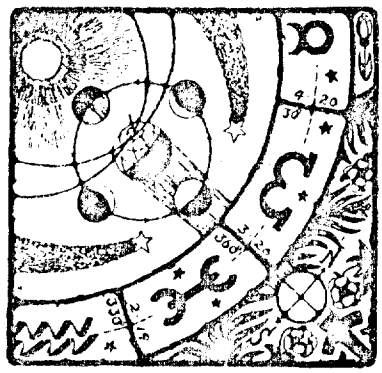

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INSIGNIFICANCE OF THE ENTEROBILIARY CIRCULATION OF LECITHIN IN MAN

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The enterobiliary circulation of intact molecules of dietary lecithin was found to be insignificant. After a single feeding of a synthetic lecithin (labeled with ^{3}H in the 1-acyl position, and with ^{14}C in the choline portion), the two isotopes were excreted in biliary lecithin at different rates. Similar amounts of radioactivity accumulated in biliary lecithin whether labeled lecithin, or labeled fatty acid and labeled choline were fed.

The aim of this study was to characterize the enterobiliary circulation of lecithin in man. It has been claimed that feeding lecithin increases the concentration of biliary lecithin and thereby enhances the solubility of cholesterol in bile.[1]

Tomkins et al.[1] fed 10 g of lecithin daily to patients for 3 days and the biliary phospholipid to cholesterol ratio increased above the value before treatment. In contrast, Thistle and Schoenfield[2] fed 20 g of lecithin daily for 14 days without changing the ratio between biliary phospholipid and cholesterol, and recently Nilsson and Scherstén[3] fed phospholipids for 6 hr to patients without observing an increased output of phospholipid in the bile.

We fed a doubly labeled synthetic lecithin and measured the radioactivity excreted in biliary lecithin to determine whether dietary lecithin is excreted intact into human bile.

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The author is indebted to Dr. H. P. Porter who helped with the care of the patients, to Miss Janet Wilson who assisted with the chemical determinations, and to Dr. P. Feigl who performed the statistical analyses.

Materials and Methods

Experimental subjects. L. M. was a 20-year-old woman. A laparotomy had been performed with a presumptive diagnosis of cholelithiasis and had led to a cholecystectomy and exploration of the common bile duct. No stones were found, but the common bile duct was described as "thickened, of small caliber." A T-tube was placed in the common bile duct. Two months later she had normal liver function tests and a normal percutaneous needle liver biopsy.

E. D. was a 65-year-old woman. Thirty-nine years previously she had had a cholecystectomy for cholelithiasis. Six weeks before this study she had had fever and pain in the right upper quadrant of the abdomen. At laparotomy, "sludge" was found in her common bile duct. Her liver was normal to inspection and palpation. A T-tube was implanted in the common bile duct. At the time of our study, her liver function tests were normal. When her biliary fistula was draining externally, she had putty-like stools which contained excess fat (93.6 mEq of fatty acid in a 72-hr specimen compared with 26.2 mEq when her enterohepatic circulation was uninterrupted; fat intake, 80 g daily). No bile salts were detected in a 45-min aspirate of duodenojejunal contents obtained ½ hr postprandially when bile salts were diverted from her intestine (lower limit of detection, 0.27 mM).

Preparation of labeled lecithin. Methyl-^{14}C-L-methionine (International Chemical and Nuclear Corporation), 0.5 mc, was dissolved in

isotonic saline, and was injected intraperitoneally into a 450-g male Sprague-Dawley rat. One hour later, the animal was killed, and the liver lipids were extracted into chloroform-methanol.[4] Liver lecithins were isolated by silicic acid chromatography,[5] and were deacylated[6] to glycerophosphoryl ^{14}C-choline which was isolated as a cadmium chloride salt. This salt was reacted[7] with the acyl chloride of 9,10-^{3}H-palmitic acid which previously had been purified by thin layer chromatography. The resulting dipalmitoyl lecithin was purified.[5] To prepare a lecithin which would be similar in chemical structure to biliary lecithins, the dipalmitoyl lecithin was converted by *Crotalus adamanteus* venom[8] to lysolecithin which was then acylated with oleoyl chloride.[9] The resulting lecithin, 1-9,10-^{3}H-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphoryl ^{14}C-choline, had a specific activity of 29.43 μc per mmole with respect to ^{3}H, and of 2.37 μc per mmole with respect to ^{14}C. It yielded a single spot after chromatography on thin layers of Silica Gel G (Brinkmann Instruments) with chloroform-methanol-water (65:25:4, by volume). Its percentage composition of phosphorus was 3.97 (theoretical, 3.90). Its specific optical rotation, $[\alpha]_D^{27}$, c = 5 g per 100 ml, chloroform-methanol (1:1, by volume), 2-dm tube, was +6.66 (reported,[10] +6.30).

Analytical methods. Biliary lecithins were obtained by chromatography on columns of silicic acid (Silic AR, CC-4 Mallinckrodt Chemical Works) as described previously.[11] Recovery of lipid phosphorus was 92%. The isolated lecithins chromatographed as a single spot on thin layer chromatoplates.

Methanolic solutions of lecithins were mixed with a dioxan-phosphor[12] and counted in duplicate in a Beckman liquid scintillation counter. An internal standard, ^{14}C-toluene or ^{3}H-toluene, was added to alternate vials.

Feces were collected continuously for 3 to 5 days after giving radioactive lecithin. The pooled feces were made up to 2000 ml with water and were blended in a paint shaker; 100 ml of the mixture were added to 1500 ml of boiling ethanol. After stirring with a magnetic chick for 2 hr, the ethanolic mixture was filtered through Whatman no. 1 filter paper. The unfiltered residue was thoroughly extracted with hot ethanol. The combined extracts were evaporated in vacuo, and the residue was dissolved in chloroform-methanol-water, 5:4:1, by volume. Portions of the resulting solution were decolorized and counted.[13] An internal standard, ^{14}C-toluene or ^{3}H-toluene, was added to alternate vials.

Experimental design. The first experiment was designed to measure the excretion rate of biliary ^{14}C and of ^{3}H. These isotopes should be excreted into the bile at identical rates if the labeled lecithin, or its hydrolytic product, 1-9,10-^{3}H-palmitoyl-*sn*-glycero-3-phosphoryl ^{14}C-choline, remained intact during their absorption from the gut or their excretion by the liver. At 8 AM, 0.584 mmole of labeled lecithin was dissolved in 5 ml of ethanol, and was fed in 240 ml of whole milk. The biliary T-tube, which up to that time had been clamped, was opened intermittently for 1-hr collections of bile over the following 4 to 5 days. During this experiment, therefore, the enterohepatic circulation of bile remained intact. The 2 patients continued to eat a constant diet which contained 80 g of fat daily.

The second experiment was designed to compare biliary excretions of radioactivity after feeding labeled lecithin, and after feeding a mixture of methyl-^{14}C-choline chloride and 9,10-^{3}H-palmitic acid. If the labeled lecithin were hydrolyzed, the subsequent biliary excretion of radioactivity might be similar to the excretion of radioactivity after ingesting labeled choline and palmitate—molecules which would be among the hydrolytic products of the labeled lecithin. Patient E. D. was placed on a constant diet which contained 80 g of fat. To ensure that the secretion of biliary lecithin had reached a plateau,[3] her enterohepatic circulation was interrupted for 69 hr prior to a meal of 0.292 mmole of labeled lecithin in 240 ml of milk. Bile and feces were collected continuously over 24 hr for the subsequent 3 days. The fecal excretion of radioactivity and the cumulative excretion of radioactivity in biliary lecithin were measured. Nineteen days later, and after 72 hr of external biliary drainage, she was given 1 μc of ^{14}C-choline with 15 μc of ^{3}H-palmitate in 240 ml of milk. The fecal excretion of radioactivity, and the cumulative excretion of radioactivity in biliary lecithin over the subsequent 3 days were measured.

Results and Discussion

In experiment 1, the labeled lecithin was nearly completely absorbed: L. M. excreted none of the ^{14}C and only 2.3% of the administered ^{3}H in a pooled, 3-day fecal collection. E. D. excreted none of the ^{14}C and only 3.7% of the ^{3}H in a pooled 5-day fecal collection. However, the fed labeled lecithin was not excreted intact into the bile. For the 1st 48 hr, patient L. M. excreted more biliary ^{3}H than

^{14}C (ratios of 116.1 at 7.5 hr, 34.4 at 24.0 hr, 42.1 at 32.8 hr, and 17.1 at 48.0 hr) relative to the ratio of 12.5 for these two isotopes in the fed lecithin; thereafter, the excretion of ^{14}C predominated. Patient E. D. excreted biliary lecithin whose ratios of ^{3}H to ^{14}C were relatively less than the ratio of these isotopes in the fed lecithin. In both subjects, the slope of the regression line for the specific activities of biliary ^{3}H was greater than the slope of the corresponding line for ^{14}C (figs. 1 and 2, table 1). Thus, the two isotopes were excreted into the bile at different rates: the fed lecithin had been hydrolyzed.

In experiment 2 (patient E. D.), only 0.3% of the absorbed labeled lecithin could have been excreted intact into the

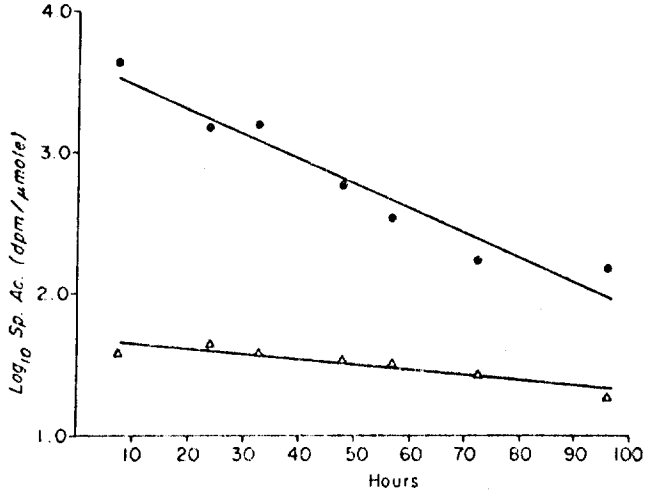

FIG. 1. Decay of ^{3}H (●——●), and of ^{14}C (△——△) in experiment 1. Patient L. M. was fed labeled lecithin, and seven 1-hr collections of bile were obtained from her biliary T-tube over the subsequent 4 days. The specific activities of biliary lecithin were determined.

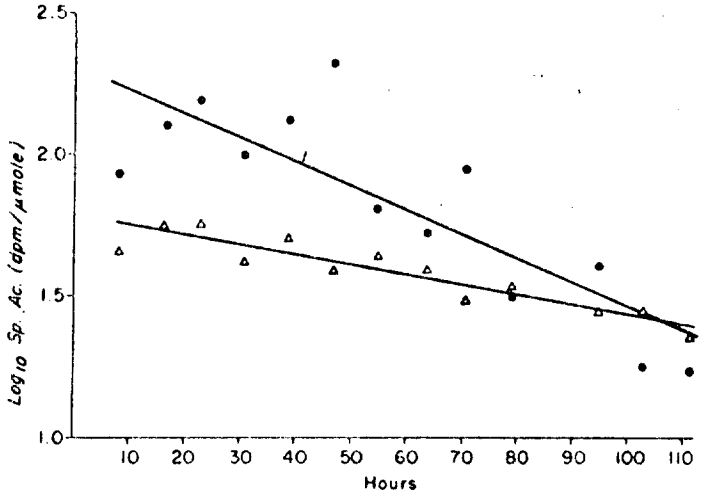

FIG. 2. Decay of ^{3}H (●——●), and of ^{14}C (△——△) in experiment 1. Patient E. D. was fed labeled lecithin, and 13 1-hr collections of bile were obtained from her biliary T-tube over the subsequent 5 days. The specific activities of biliary lecithin were determined.

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TABLE 1. *Statistical data in experiment 1*

Patient	Isotope	Regression equation	$t_{\frac{1}{2}}$	95% confidence interval for slope[c]	Difference between slopes
			hr		
L. M.[a]	^{3}H	$y = 3.66 - 0.0174x$	105	-0.0228 to -0.0122	$P < 0.01$
	^{14}C	$y = 1.69 - 0.00373x$	226	-0.00562 to -0.00189	
E. D.[b]	^{3}H	$y = 2.32 - 0.00860x$	135	-0.0124 to -0.00478	$P < 0.05$
	^{14}C	$y = 1.80 - 0.00348x$	258	-0.00441 to -0.00254	

[a] Data illustrated in Figure 1.
[b] Data illustrated in Figure 2.
[c] Reference 14.

bile (table 2); less than 5% of the choline ^{14}C of the fed lecithin was used for the synthesis of biliary lecithin. Similar labeling of biliary lecithin was found after feeding radioactive choline and palmitic acid—molecules which would have presumably been among the hydrolytic products of the labeled lecithin. These values are minimal estimates of the utilization of the fed lecithin, or its hydrolytic products, for synthesis of biliary lecithin. We were unable to obstruct the distal portion of the common bile duct[15] to ensure a complete biliary fistula. Furthermore, the biliary excretion of lecithin was undoubtedly reduced because the enterohepatic circulation of bile salts was interrupted.[3, 15]

These data indicate that dietary lecithin is extensively hydrolyzed during its absorption from the gut and its transport to the liver. Its hydrolytic products apparently enter different precursor pools which may then contribute to the synthesis of biliary lecithin. Whether supplemental dietary lecithin induces an increased excretion of biliary lecithin could be answered by analyzing quantitative fractions of bile without disturbing the enterohepatic circulation.[15] The results of the present study indicate that the enterobiliary circulation of intact molecules of lecithin is insignificant.

TABLE 2. *Recovery of radioactivity in experiment 2*[a]

Material fed	Bile volume	Fecal excretion		Recovery in biliary lecithin	
		^{3}H	^{14}C	^{3}H	^{14}C
	ml in 3 days	*% fed radioactivity*		*% absorbed radioactivity*	
Labeled lecithin	548	9.5	0.0	0.3	4.8
^{3}H palmitate + ^{14}C choline	488	31.7	4.2	1.1	4.5

[a] Patient E. D. was fed either labeled lecithin (1-^{3}H palmitoyl, ^{14}C-choline), or ^{3}H-palmitate and ^{14}C-choline when her bile was diverted. Feces and bile were collected for the subsequent 3 days. Radioactivity in the pooled sample of feces and the cumulative radioactivity in biliary lecithin were determined.

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Components of "Soybean Lecithin"[1]

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DESPITE years of production and use, information as to the composition of "soybean lecithin" is surprisingly meager. Recent texts and papers repeat the statement that soybean phosphatides consist of approximately 35% lecithin[3] and 65% cephalin (1,2,3,). While lecithin is readily prepared in high purity from the alcohol-soluble fraction through the cadmium salt precipitation (4,5), the cephalin fraction or alcohol-insoluble portion has been poorly characterized. Phosphatides other than lecithin and cephalin are known to be present in soybean oil. McKinney, Jamieson, and Holton (6) have reported the presence of diamino-monophosphoric acid and monamino-diphosphoric acid phosphatides and of a glycosidal lecithin complex. Levene and Rolf (7) have reported a fraction which they describe as resembling cuorin. They regarded this fraction as being formed by partial hydrolysis of lecithin and cephalin, but in the light of present knowledge it seems probable that it contained a large amount of inositol-containing phosphatides. As early as 1930 Klenk and Sakai (8) isolated inositol and inositol monophosphoric acid from soybean cephalin hydrolyzates, but it was not until 1943 that Woolley (9) isolated an inositol-containing phosphatide which he called lipositol. More recently, in 1947, Folch (10) reported the presence of a phosphatide containing both inositol and glycerol. However, insufficient information is available as to the number and composition of inositol-containing phosphatides to permit an estimate of the composition of soybean "lecithin."

Lack of adequate methods for fractionation of the complex mixture of soybean phosphatides has hindered study of their composition. Adsorption and partition chromatography have been found inapplicable (11,12). The technique of "countercurrent distribution," developed by Craig (13,14) and applied with outstanding success in the separation of the penicillins, offered a new method of fractionation. This technique has been highly effective, and some preliminary analyses of soybean phosphatides obtained by this procedure are presented.

Analytical Methods

The procedures used for the determination of nitrogen, phosphorus, choline, amino nitrogen, sugar, and inositol were dictated in part by the size of samples available from the distribution apparatus and in part by complexity of the materials being analyzed.

Total nitrogen was determined by the micro-Kjeldahl procedure. Phosphorus determinations reported in Table I were obtained gravimetrically as ammonium phosphomolybdate. Owing to the small size of

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[3] Except when enclosed by quotation marks to denote the common usage in the trade, the word lecithin refers specifically to phosphatidyl choline.

TABLE I

Analysis of Phosphatide Preparations

Preparation	Per Cent of total weight	Nitrogen	Phosphorus	Molar ratio	Choline nitrogen	Amino nitrogen	Sugar	Inositol
		%	%	P/N	%	%	%	%
Crude phosphatides	100	1.24	3.37	1.27	0.42	0.62	3.96	4.18
Fraction I	50.7	1.65	3.54	0.97	0.81	0.62	2.38	0.26
Fraction II	4.4	1.09	2.87	1.19	0.04	0.81	7.08	2.80
Fraction III	6.6	1.24	3.10	1.12	0.17	1.14	5.52	1.66
Fraction IV	38.3	0.79	1.30	1.89	0.00	0.62	5.15	11.3

the samples obtained from countercurrent distribution, phosphorus in the samples from this apparatus was measured spectrophotometrically by Burmaster's method for total phosphorus (15). In the oil itself phosphorus was determined by a modification of the method of Truog and Meyer (16). Both of the last mentioned methods involve the measurement of optical density of the molybdenum blue complex. Choline was measured by the reineckate method of Glick (17).

Amino nitrogen in the preparations in Table I was determined by the micro method of Van Slyke (18) after refluxing the sample for 24 hours with $2N$ H_2SO_4, and filtering. It was found that the Burmaster periodate oxidation method (19) gave results agreeing with those obtained by the Van Slyke method on the alcohol-soluble phosphatides. Consequently, the Burmaster method was used for the samples from the countercurrent distribution of the alcohol-soluble fraction because of the much smaller sample required. For convenience in handling the large number of samples from this distribution these samples were hydrolyzed with $2N$ H_2SO_4 at 120°C. in an autoclave overnight. In dealing with the alcohol-insoluble phosphatides, the Burmaster method gave results lower than the Van Slyke method, and the Burmaster method was therefore not applicable to this fraction. This is in agreement with the observation of Burmaster (20) that low amino nitrogen values were obtained in fractions of calf brain phosphatides containing inositol, when compared with values obtained by the Van Slyke method.

Sugar was determined by first hydrolyzing the phosphatides for approximately seven hours with $.6N$ H_2SO_4 on a steam bath. After the hydrolyzate was filtered and neutralized, sugar was measured by the method of Stiles, Peterson, and Fred (21). Since Woolley has reported that galactose is present in soybean lipositol (9), all sugar was uniformly calculated as galactose although other sugars were probably present.

Inositol was estimated by a modification of the Atkin, Schultz, Williams, and Frey (22) microbiological method for the assay of pyridoxin. Samples were hydrolyzed with 20% HCl at 120°C. for 16 hours prior to assay.

Preparation of Fractions

The phosphatides used in this work were separated from the oil by passing steam into 10 liters of commercial crude hexane-extracted soybean oil and allowing the hydrated phosphatides to precipitate as a sludge. After removal of this sludge, the steaming, settling, and removal steps were repeated twice. The combined sludges were centrifuged to separate the phosphatides from oil and water. The crude phosphatides were dissolved in diethyl ether, and the resulting emulsion was broken by the addition of sodium sulfate. After evaporation of the ether solution to small volume, the phosphatides were precipitated from the oil with acetone. The precipitate obtained was extracted twice by stirring with acetone, redissolved in ether, and reprecipitated with acetone. The precipitate was washed with successive portions of acetone until the preparation became waxy. Four more extractions were made with the aid of a Waring Blendor. The total weight of the phosphatides was 124 grams. Analytical data on this crude phosphatide preparation are shown in Table I.

Since the crude oil contained 0.072% phosphorus and, after steaming, 0.0035% phosphorus, 94% of the phosphorus in the crude oil was removed and 63% was recovered in the phosphatide preparation.

The crude phosphatides were first separated into alcohol-soluble and alcohol-insoluble fractions. Of the crude material 114 grams were extracted with 350-ml. portions of absolute alcohol six times in a Waring Blendor. The alcohol-soluble portion was further fractionated by removing the solvent under vacuum at 50°C. and again adding absolute alcohol. The alcohol was evaporated from the clear supernatent solution, leaving 55 grams, which are designated in Table I as Fraction I. A small portion, 4.8 grams, which did not redissolve in the alcohol, is designated as Fraction II. The alcohol-insoluble portion was extracted twice in the Blendor with absolute alcohol warmed to 50°C. This alcohol solution was evaporated under reduced pressure at 50°C., leaving 7.1 grams residue designated as Fraction III. The alcohol-insoluble material remaining weighed 41.5 grams and is designated as Fraction IV. (Analytical data on these fractions is also given in Table I.)

The phosphatide fractions were protected from oxidation by keeping them under carbon dioxide as much as possible during their preparation and by storing them at —16°C. in a vacuum desiccator.

Countercurrent Distribution of Fractions I and IV

In principle, the countercurrent distribution apparatus consists of a series of separatory funnels, each funnel containing a pair of immiscible solvents. In the actual apparatus the separatory funnels consist of a series of tubes or holes bored in a cylindrical metal block, and the upper phase of each tube may be readily transferred to the adjacent tube containing a lower phase, thus achieving countercurrent movement of solvents.

To resolve a mixture of solutes, the mixture is introduced into one of the tubes. By shaking and allowing the solvents to separate, each solute of the mixture is distributed between the two solvents in the tube according to its partition coefficient. The alternation of countercurrent movement and mixing of solvents tends to separate individual solutes of the mixture into particular tubes since the rate of movement of each solute through the tubes depends upon its specific partition coefficient. When the desired

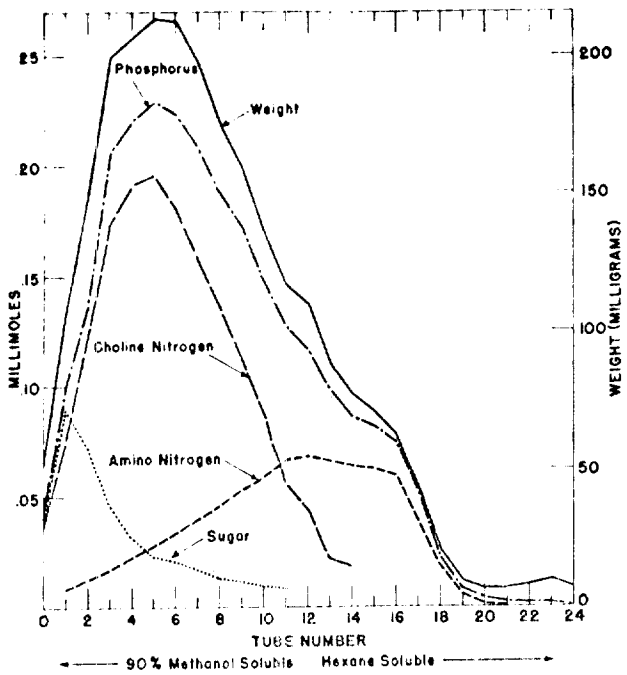

FIG. 1. Results of the countercurrent distribution of the alcohol soluble fraction.

number of transfers have been performed, the contents of the individual tubes are removed and, after evaporation, the residues are weighed and analyzed for constituents. Choice of the pair of immiscible solvents to be used is made with regard to the partition coefficients of the compounds to be fractionated and with regard to the rapidity with which the emulsion breaks. Because of limitations imposed by the design of the distribution apparatus, it is also critical that the volume of the lower phase does not change on introduction of the sample.

For work with the alcohol-soluble fraction, hexane and 90% methanol were found to be a suitable pair of solvents. Fraction I was dissolved in hexane, and an aliquot containing 197 mg. was distributed between hexane and 90% methanol in the apparatus. After the sample had been carried through the 24 distributions which can be performed in our 25-tube apparatus, it was found that very little material had reached tube 24. In order to obtain better fractionation six additional distributions were applied using the withdrawal technique described by Craig (13). In this technique the contents of tube 24 are withdrawn and replaced by fresh solvent after each distribution.

The distribution operation just described was repeated four times, and the contents of the respective tubes from each experiment were combined. After the solvents were evaporated, the residues were left in a vacuum desiccator until constant weights were obtained. The weights of the fractions are shown plotted against tube number in Fig. 1. Weights of fractions 25-30 are small and are not plotted on the graph. Phosphorus, choline nitrogen, amino nitrogen, and sugar were determined on the fractions wherever a sufficient amount of material was obtained. These results were calculated as millimoles and are also shown in Fig. 1.

For the countercurrent distribution of the alcohol-insoluble fraction, hexane and 95% methanol were found to comprise a suitable pair of solvents. A solution of Fraction IV was made with hexane, and an aliquot containing 500 mg. was distributed between the two solvents in one of the tubes. After the distribution the solvents were badly emulsified, and the contents of this tube were removed and centrifuged to give a clear upper and lower layer with some insoluble material at the interface. The entire sample was then returned to the apparatus with care to avoid re-emulsification. In the succeeding distributions this procedure for breaking emulsions was unnecessary. After the 24 distributions had been performed, the fractions were removed from each tube. This complete distribution operation was repeated as just described. The contents from the respective tubes were then combined and the solvent was evaporated. The residues were left in a vacuum desiccator until they reached constant weight. Phosphorus, total nitrogen, sugar, and inositol were determined wherever a sufficient amount of material was obtained. The analytical values expressed as millimoles are plotted against tube number in Fig. 2 along with the total weight of the fractions in milligrams.

Discussion

Choline- and inositol-containing phosphatides are separated from each other quite satisfactorily on the basis of solubility in absolute alcohol as shown in Table I. However, amino nitrogen constituents are present in both the alcohol-soluble and alcohol-insoluble fractions. Since only a trace of inositol is present in Fraction I and since this fraction has a phosphorus to nitrogen ratio of .97:1, it is concluded the amino nitrogen constituent of the alcohol-soluble fraction is cephalin. Fraction IV is estimated to contain 11% inositol. Also this fraction has a phosphorus-to-nitrogen ratio of 1.89:1, and a phosphorus-to-inositol ratio of 1.70:1. Since cephalin has a phosphorus-to-nitrogen ratio of 1:1, it appears that only a small amount of cephalin (approximately 5%) remains in the alcohol-insoluble fraction. This is contrary to the common idea that lecithin and cephalin are separated on the basis of their solubility in alcohol.

The results of the countercurrent distribution of the alcohol-soluble fraction (Fraction I) are given in Fig. 1. It is apparent from this data that lecithin, cephalin, and a sugar-containing compound are present. When a pure compound is distributed among immiscible solvents in the countercurrent distribution apparatus and the amount of material in each tube is plotted against the tube number (as in Figs. 1 and 2), a Gaussian distribution curve should be obtained (13). In the analysis of a mixture the curve will be the sum of the curves for each component. Thus the weight curves in Figs. 1 and 2 represent the sum of the weights of the components in the mixture. The phosphorus curves represent the sum of the phosphorus contents of all the compounds.

The weight and phosphorus curves of Fig. 1 each show a peak coincident with the peak in the choline nitrogen curve and a hump corresponding to the peak in the amino nitrogen curve. The shape of the choline and amino nitrogen curves are that which might be expected for groups of closely related compounds, such as the lecithins and cephalins containing fatty acids differing in degree of unsaturation and chain

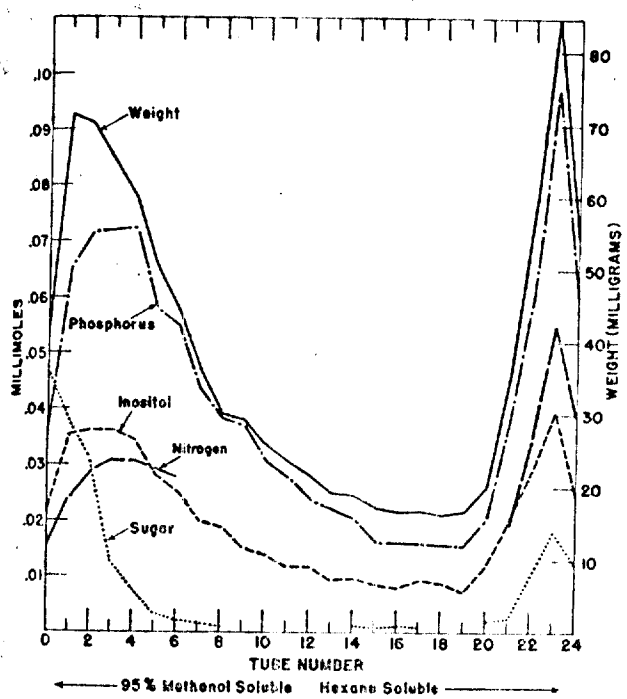

FIG. 2. Results of the countercurrent distribution of the alcohol-insoluble fraction.

length. However, the concentrations of solutes present in these distributions is sufficiently high that partition coefficients may be changing with concentration (23) and in part account for the deviation from the theoretical type curve. It is also probable that complexes exist between lecithin and cephalin and between the phosphatides and the sugar components. Such association effects or mutual solubility effects would also distort the curve.

The countercurrent distribution data for Fraction IV, the "cephalin" or alcohol-insoluble fraction, are given in Fig. 2. It is immediately apparent that the weight curve is composed of two peaks, one in the hexane-soluble portion of the curve and the other in the methanol-soluble portion. The shape of the curve for the hexane-soluble material approaches the theoretical for a single substance while the shape of the curve for the methanol-soluble components is sharp on the left but trails to the right, the latter being indicative of a mixture of related compounds. The most surprising part of the data is that within the limits of experimental error the phosphorus-to nitrogen-to-inositol ratio of 2:1:1 is present in all the tubes, both in the hexane-soluble phosphatides and in the 95% methanol-soluble phosphatides.

The sugar content at the left of the curve is high, 19% of the dry weight in tube 0, and decreases to 0.6% in tube 8. Sugar is also found in the peak at the right of Fig. 2 but in smaller amount. In tube 23 there is 3.9% or one mole of sugar to 5.4 moles of phosphorus. It appears doubtful, therefore, that sugar is a constituent of these phosphatides. Rather, it may be complexed or held by secondary valence forces.

No chemical evidence has yet been obtained which would account for the varying solubility of the inositol phosphatides. The presence of glycerol (10) or differences in number and unsaturation of associated fatty acids may possibly account for the difference in properties. Whether the sugar compounds are present in a sugar phosphatide complex or whether sugar is a component of the inositol phosphatides, the high sugar fractions are more alcohol-soluble, as would be anticipated. The phosphorus-to-nitrogen and phosphorus-to-inositol ratios and the low sugar content of certain fractions are not in agreement with data of Woolley (9) and Folch (10) on the inositol-containing phosphatides from soybean lecithin. Further study is necessary before conclusions can be drawn as to the number and structure of inositol-containing phosphatides.

A rough estimate of the composition of the crude phosphatides used in this work can be calculated from the analytical data in Table I and the information obtained by countercurrent distribution. It should be pointed out that the crude phosphatides examined contained only 63% of the phosphorus of the crude oil. This estimate is distinctly tentative and will be altered as more information becomes available. From the choline nitrogen content of the crude phosphotides, lecithin is calculated as 24% if the molecular weight of 278.3 is assumed for the fatty acids as found by Thornton, Johnson, and Ewan (5).

If the inositol phosphatides are considered as containing one nitrogen for each inositol, and if the remainder of the non-choline nitrogen is assigned to cephalin, an estimate of cephalin content can be made. Assuming the same molecular weight for the fatty acids as was used with lecithin, the crude phosphatides are estimated to contain 25% cephalin.

In a calculation of the amount of inositol-phosphatides some estimate of the percentage of inositol in the pure inositol-phosphatides is necessary. At least two inositol-containing phosphatides appear to be present. However, the inositol and weight curves of Fig. 2 indicate that each tube contains approximately the same percentage of inositol. If the inositol content of Fraction IV is corrected for sugar and cephalin impurities, the inositol containing phosphatides are estimated to contain 12.5% inositol. [Woolley's lipositol was reported to contain 16% inositol (9).] Based on this figure of 12.5%, the crude phosphatides contain 33% inositol phosphatides.

The values calculated above are percentage by weight in the crude phosphatides. The remainder of the crude phosphatide preparation, approximately 17%, can only partially be accounted for as sugars, sterol glycosides, etc. Excluding these materials, 29% of the phosphatides present in this crude phosphatide preparation is lecithin, 31% is cephalin, and 40% is inositol-containing phosphatides. This agrees with the accepted value for lecithin, but it appears that much of the phosphatides formerly considered to be cephalin are actually inositol-containing phosphatides.

Summary

The acetone-insoluble material from soybean "lecithin" has been fractionated by submitting alcohol-soluble and alcohol-insoluble portions to countercurrent distribution between hexane and methanol. The alcohol-soluble portion was found to contain lecithin, cephalin, and sugars or glycosides; the alcohol-insoluble portion was separated into two major inositol-containing phosphatides and sugars or glycosides. While the commonly accepted value of 30-35% for lecithin in the phosphatides was confirmed, it appears that the accepted value of 65% of cephalin

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needs revision. The approximate composition for one sample of soybean phosphatides is estimated to be 29% lecithin, 31% cephalin, and 40% inositol-phosphatides.

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EFFECT OF LECITHIN ON THE CONDITIONED REFLEX ACTIVITY OF RATS

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A number of studies (A. Ya. Danilevskii, E. K. Prikhod'kova, A. M. Vorob'ev, I. N. Zhuravlev, et al.) have shown that lecithin causes significant changes in the activity of the central nervous system.

A. I. Makarychev and M. A. Sergeeva have shown that lecithin also exerts a considerable effect on the conditioned reflex activity of the brain cortex of dogs.

The present article is concerned with a study of the conditioned reflex activity of rats, using the method of defensive conditioned reflexes (electrostimulation in a 3-chamber passage).

An electric current of minimum strength from the city power supply system was used as the unconditioned stimulus, inducing a threshold stimulation, while light and sound from a bell were used as the conditioned signals. In developing a conditioned reflex in rats, the electric current was switched on in the floor of the starting chamber (where the rat was located) and in the floor of the middle chamber for 4-6 seconds until the rat ran over into the third section of the passage where no current was turned on. After conditioned reflexes were established, current stimulation was occasionally used (for a duration of 1 second) to strengthen the reflex.

Before the test, the irritability threshold was determined in 24 rats and electroencephalograms were recorded, in order to subdivide animals with a different irritability threshold and a different bioelectric activity of the brain into 3 groups, namely one control group and two test groups (with 8 rats in each group).

All rats received a food mixture consisting of casein, corn starch, fat, salts and vitamins.

Lecithin was given per os to the test animals, in a dose of 1 mg/200 g BW to one group, and in a dose of 25 mg/200 BW to the second group.

After the different doses of lecithin had been given for 3 weeks, the development of a conditioned reflex to light and sound (bell) was started; the study included the time at which the conditioned reflex was manifested, its establishment (consolidation), and any changes in the already established reflex. The test period, with a daily administration of lecithin, lasted 40 days. At the end of the study, the brain biocurrents were recorded.

The results obtained showed that lecithin causes changes in the irritability (excitability) of the brain cortex, which is increased with small doses (1 mg/200 g) and decreased (lowered) with large doses (25 mg/200 g).

In control rats (which did not get lecithin), the conditioned reflex activity did not change during the testing period. Also, no changes were noted in the magnitude of the irritability threshold and the bioelectric activity of the brain cortex.

In control rats, positive defensive conditioned reflexes were generated at the 20-30th combined stimulus, and discrimination was established at the 4-5th combined stimulus. The animals behaved quietly during the intervals between stimulations.

A different pattern was noted in the test groups. In rats which received small doses of lecithin, the irritability threshold decreased by a factor of 1.5 to 2, and conditioned reflexes were generated at the 15-20th combined stimulus. In 4 animals of this group, a continuous conditioned running about the passage was noted (rats nos. 11, 12, 13, and 10), while in 4 other rats the reflex was expressed more strikingly in a conditioned squeak, the running about was unsystematic, although almost always a reaction to time

was noted, namely, at the moment the conditioned signal was given, the animals ran up to the opening of the middle chamber.

The positive conditioned reflexes thus developed were quite stable, they became extinct (vanished) in most rats only when the stimuli were applied without any reinforcement during the course of 10 or more times. Inhibiting reflexes developed in a normal manner. The rats were very mobile; during intervals between stimulations they frequently ran along the passage (6-7 times during a 3-minute interval). The brain biocurrents at the end of the test period were different from those recorded prior to administration of lecithin, and were characterized by waves with a more frequent rhythm (?* - and β - like waves).

Thus, lecithin in small doses increased the excitability of the brain cortex.

The use of lecithin in a dose of 25 mg/200 g gave the opposite results, namely a lowering of the excitability of the brain cortex. The irritability threshold in animals of this group increased 1.5 times during the test period. Conditioned reactions were expressed only in the form of squeaking, and the rats ran through the entire passage mostly after the action of the unconditioned stimulus had ceased. Upon stimulation by the electric current, the rats remained in the starting and middle chamber, or, after having run halfway through the passage, they returned again to the starting chamber. During the test, an increased diuresis was noted in the animals (possibly as a result of a weakening of the function of the brain cortex). In rats of this group, the brain biocurrents also exhibited a change, but a different one than in the rats of the first test group. At the end of the test, waves of a slow rhythm were noted on the encephalogram, and in 2 rats (nos. 4 and 2) peak-shaped (spiked) waves appeared.

* symbol illegible

Thus, the results of this investigation have shown the effect of different lecithin doses on the process of development of conditioned reflexes in rats: at a small dose, the conditioned reflexes of test rats developed more quickly than those of controls, were stable and became extinct (vanished) with difficulty. With a large dose of lecithin, it was not possible to develop a conditioned reflex, which would make the rats run along the passage.

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ВЛИЯНИЕ ЛЕЦИТИНА НА УСЛОВНОРЕФЛЕКТОРНУЮ ДЕЯТЕЛЬНОСТЬ КРЫС

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Из лаборатории высшей нервной деятельности (зав — проф. А. И. Макарычев) Института питания АМН СССР, Москва

Рядом работ (А. Я. Данилевский, Е. К. Приходькова, А. М. Воробьев, Н. Н. Журавлев и др.) доказано, что лецитин существенно изменяет деятельность центральной нервной системы.

А. И. Макарычев и М. А. Сергеева показали, что лецитин оказывает значительное влияние и на условнорефлекторную деятельность коры головного мозга собак.

В настоящей работе изучалась условнорефлекторная деятельность у крыс методом оборонительных условных рефлексов (электрораздражение в 3-камерном коридоре).

Безусловным раздражителем служил электрический ток от городской сети минимальной силы, вызывающий пороговое раздражение; условными сигналами — свет и звонок. При выработке у крыс условного рефлекса электрический ток включали в пол исходной камеры (где помещали крысу) и в пол средней камеры на

4—6 секунд до перебежки в 3-ю, бестоковую, часть коридора. После упрочения условных рефлексов раздражение током применялось изредка (длительностью 1 секунду) для подкрепления рефлекса.

Перед опытом у 24 крыс был определен порог возбудимости и произведена запись электроэнцефалограмм с целью распределения животных с различной величиной пороговой возбудимости и различной характеристикой биоэлектрической активности мозга в три группы — одну контрольную и две подопытных (по 8 крыс в каждой).

Все крысы получали пищевую смесь, состоящую из казеина, маисового крахмала, жира, солей и витаминов.

Подопытным животным лецитин вводили per os одной группе — по 1 мг на 200 г веса животного, другой — по 25 мг на 200 г.

После 3-недельной дачи различных доз лецитина была начата выработка условного рефлекса на свет и звонок — изучалось время проявления условного рефлекса, его закрепление и изменения уже установившегося рефлекса. Опытный период с ежедневной дачей лецитина продолжался 40 дней. В конце исследования производилась запись биотоков мозга.

Полученные результаты показали, что лецитин изменяет возбудимость коры мозга: в малых количествах (1 мг/200 г) он повышает ее, а в больших (25 мг/200 г) — понижает.

У крыс контрольной группы (не получавших лецитина) условнорефлекторная деятельность за время исследования не изменялась. Не наблюдалось также изменений величины порога возбудимости и биоэлектрической активности коры головного мозга.

Положительные условные оборонительные рефлексы у крыс контрольной группы вырабатывались на 20—30-м сочетании, а дифференцировка — на 4—5-м сочетании. В перерывы между раздражениями животные вели себя спокойно.

Иная картина наблюдалась в подопытных группах. У крыс, получавших малое количество лецитина, порог возбудимости уменьшался в $1^1/_2$—2 раза, условные рефлексы вырабатывались на 15—20-м сочетании. У 4 животных этой группы отмечалась безотказная условная перебежка по коридору (крысы № 11, 12, 13 и 10), а у четырех других рефлекс более ярко проявлялся в условном писке, перебежка была несистематическая, хотя почти всегда наблюдалась реакция на время — к моменту подачи условных сигналов животные подбегали к отверстию средней камеры.

Выработанные положительные условные рефлексы оказались весьма устойчивыми, они угасали у большинства крыс только после применения раздражителей без подкрепления в течение 10 и более раз. Тормозные рефлексы вырабатывались нормально. Крысы были очень подвижны: в перерыве между раздражениями они часто бегали по коридору (6—7 раз за 3-минутный интервал). Биотоки мозга в конце опытного периода отличались от биотоков, записанных до дачи лецитина, и характеризовались волнами с более частым ритмом (- и β-подобным).

Таким образом, лецитин в малых дозах повышал возбудимость коры головного мозга.

Применение лецитина в дозе 25 мг/200 г привело к обратным результатам — понижению возбудимости коры головного мозга. Порог возбудимости у животных этой группы в период опыта повышался (в $1^1/_2$ раза). Условные реакции проявлялись только в форме писка — крысы пробегали через весь коридор большей частью после прекращения действия безусловного раздражителя. При раздражении электрическим током они задерживались в исходной и в средней камере или, добежав до середины коридора, снова возвращались в исходную камеру. Во время опыта у них наблюдался повышенный диурез (возможно, в результате ослабления функции коры мозга). Биотоки мозга у крыс этой группы также изменялись, но иначе, чем у крыс первой подопытной группы. К концу опыта на электроэнцефалограмме отмечались волны с медленным ритмом, а у 2 крыс (№ 4 и 2) появились пикообразные волны.

Таким образом, результаты проведенного исследования показали влияние различных доз лецитина на процесс выработки условных рефлексов у крыс: при малой дозе условные рефлексы у подопытных крыс вырабатывались быстрее, чем у контрольных, были стойкими и угашались с трудом. При большой дозе лецитина условного рефлекса на бег по коридору выработать не удалось.

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The Effect of Phosphatides on Utilization of Vitamin A and Carotene.

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The problem of the utilization of carotene and vitamin A as influenced by the oil or fat in the diet has been investigated repeatedly during recent years.

Sherman[1] studied the effect of various oils on the utilization of carotene and found that the addition of 0.1 ml of cottonseed oil daily and especially soybean oil to the diet greatly improved the growth response of vitamin A deficient rats receiving 1 and 2 μg carotene daily.

Quackenbusch, Cox and Steenbock[2] reported that tocopherol promoted the response to carotene and concluded that tocopherol functioned in the intestinal tract as an antioxidant.

Sherman[3] reported that carotene was destroyed in the intestinal tract in the absence of tocopherol.

Quackenbusch, Cox and Steenbock[4] provided further evidence that tocopherol is essential for the utilization of carotene in the intestinal tract but reported even better results with a soybean oil distillate.

[1] Sherman, W. C., *J. Nutrition*, 1941, **22**, 153.

[2] Quackenbusch, F. W., Cox, R. P., and Steenbock, H., *J. Biol. Chem.*, 1941, **140**, civ.

[3] Sherman, W. C., *Fed. Proc. Soc. Biol. Chem.*, 1942, **1**, 134.

[4] Quackenbusch, F. W., Cox, R. P., and Steenbock, H., *J. Biol. Chem.*, 1942, **145**, 169.

As it is known that the antioxidant activity of tocopherol *in vitro* is greatly influenced by cephalin[5] it seemed advisable to investigate this problem further. The evidence presented by our experiments indicates that this synergism between cephalin and tocopherol exists also *in vivo* but that other factors may be involved in the utilization of carotene and vitamin A by the rat.

Experimental. Female albino rats, Sherman strain, from our laboratory colony, 28 to 30 days old, weighing approximately 60 to 66 g, were kept in individual metal cages provided with raised screen floors. The basal diet used had the following percentage composition: casein Labco, 20; cerelose, 72; salts, Osborne and Mendel, 4; cottonseed oil, 4. This diet was supplemented with thiamine 2 mg, riboflavin 4 mg, pyridoxine 4 mg, nicotinic acid 1 mg, choline 500 mg and 200 mg of Viosterol per kilogram of diet. Synthetic calcium pantothenate was given orally, 100 μg daily. The rats were weighed daily during the depletion period and 3 times a week during the 4-week period when the test substances (phosphatides and soybean oil) were included in the diet. The purified diets and mixtures were freshly prepared each week and stored at 4°C. Food consumption was determined.

Discussion. Although our basal diet was similar to the diet used by Sherman, Steenbock, *et al.*, we failed to get the expected growth response with the rats receiving 2 U.S.P. units carotene or vitamin A and a diet containing 4% cottonseed oil which should have been sufficient to provide the necessary tocopherol. However, our diet differed from those mentioned before in that both authors used yeast or rice bran extract as a source of the B complex in their diet while we used the synthetic vitamins mentioned above. The striking response of our rats to 1% soybean phosphatides,* and to a certain degree to 0.3% soybean oil, indicates the presence of an unknown factor, possibly also present in yeast or the B complex and essential for the utilization of vitamin A and that this factor is not present in cottonseed oil.

That factors of the B complex may be essential for the utilization of vitamin A was proved by Popper[6] who showed that choline deficiency interferes with vitamin A utilization. However, in our experiments, enough choline was provided to exclude this possibility. It may be noted that the presence of a hypothetical factor for the utilization of vitamin A and carotene has been suspected before[7] and it was claimed that palm kernel meal, coconut cake and acetone-extracted herring roe are rich in this factor.

It can be seen from Chart 1 that the vitamin A-depleted rats gave essentially the same type of growth response with 2 units of carotene as with 2 units of vitamin A (cod liver oil) when 1% of soybean phosphatides was added to the diet. As these soybean phosphatides contain considerable amounts of soybean oil (up to 30%) two groups of rats were given the equivalent amount of soybean oil (0.3%) instead of the phosphatides in the diet. Although the rats receiving soybean oil plus 2 units of vitamin A showed a gain in weight, they failed to equal that obtained with the phosphatides. After the third week the rats receiving soybean oil instead of soybean phosphatides started to lose weight while those receiving phosphatides continued to gain. Even more striking was the fact that the animals receiving phosphatides were in much better condition at the end of the 4-week test period than the rats receiving only soybean oil. The fur of the soybean oil rats appeared shaggy and dry while that of the phosphatides rats was sleek and glossy.

The above experiments were repeated substituting 4% soybean phosphatides in the place of 1% phosphatides in the diet. The increased intake of soybean phosphatides did not appreciably change the gain in weight.

In addition, two groups of vitamin A de-

[5] Swift, C. E., Rose, W. G., and Jamieson, G. S., *Oil and Soap*, 1942, **19**, 176.

* We are indebted to the American Lecithin Co., Elmhurst, L.I., N.Y., for furnishing us with commercial soybean phosphatides. These soybean phosphatides are composed of about 20 to 25% lecithin, 25 to 30% cephalin fraction, including some carbohydrate, about 15% inositol phosphatides and a carrier of 30% soybean oil.

[6] Popper, H., and Chinn, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 202.

[7] Morton, R. A., *Ann. Rev. Biochem.*, 1942, **11**, 371.

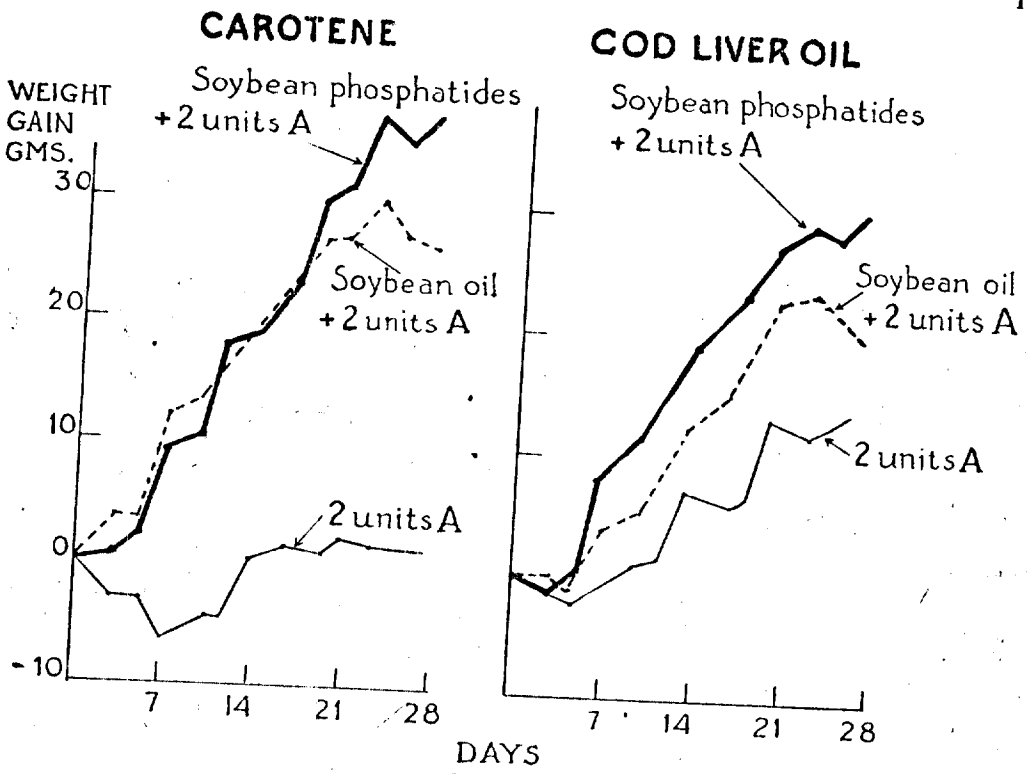

CHART 1.

pleted rats were given soybean phosphatides without vitamin A. These rats lost weight even more rapidly than those receiving the basal diet without vitamin A. This response may be due to the strong lipotropic effect of soybean lecithin.

Summary. Vitamin A-depleted rats fed a vitamin-free diet, supplemented with synthetic B vitamins and 2 U.S.P. units of vitamin A in the form of carotene, failed to gain weight in spite of the presence of 4% cottonseed oil in the diet. Satisfactory growth was obtained when soybean phosphatides (1%) were added to the diet. An amount of soybean oil (0.3%) equivalent to that contained in the soybean phosphatides used also promoted growth but to a lesser degree. Rats fed the vitamin A-deficient diet supplemented with soybean oil and 2 units of vitamin A appeared in poor condition and began to lose weight after the third week.

Substitution of cod liver oil in place of carotene as the source of vitamin A in the diet, with addition of soybean oil or soybean phosphatides resulted in a growth response similar to that obtained with carotene. However, cod liver oil alone also caused some gain in weight.

In the presence of soybean phosphatides, carotene was as well utilized as vitamin A under the conditions of our experiment.

Use of Soya Lecithin Will Save Scarce Fats

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This ingredient will also extend the stability of fats, will reduce the amount of egg yolk required in ice cream, will stabilize vitamin A potency and extend vitamin B_1 activity, and can be employed to cut costs and improve quality in the confectionery, baking and ice cream industries

BECAUSE of economic dislocations arising from the present world war, soya lecithin is acquiring an increased importance in improving the quality, extending the period of stability, and cutting the costs of foodstuffs. Foods containing fats, egg yolks and vitamins A and B_1 are particularly affected. Savings of important fats may be had by using lecithin in the confectionery, chocolate, biscuit and bread making industries. The period of stability of the edible fats themselves may be lengthened for long storage or export by a trace of soya lecithin. In products such as ice cream, the supply of egg yolk can be extended effectively by a mixture of lecithin and glyceryl monostearate.[1] German military emergency rations contain lecithin.[2]

Utilization of all grades of flour in macaroni products is possible by inclusion of 1 percent of lecithin. Stabilization of vitamin A potency and extension of vitamin B_1 activity are also accomplished by adding soya lecithin. Because of their excellent properties, vitamin-fortified mixtures of lecithin-containing margarine and dairy butter are popular in Europe, even in peacetime.

As known commercially, soya lecithin, or simply "lecithin," is the phosphatide constituent of the soy bean. It serves as an excellent interface agent (a reducer of friction or attraction between particles) in food mixtures, and as an antioxidant. Most prepared foods are random or unorganized systems of water- and fat-containing constituents, often in the finely divided or collodial state. Most frequently we encounter a glyceride intermixed with protein or carbohydrate matter in varying degrees of dispersion, with or without water. In such foods, lecithin shows its colloidal properties at the contact points of the suspension or mixture. When lecithin is dispersed in these foods its molecules proceed at once to the fat-water interfaces, arrange themselves in an orderly single-molecule layer, and greatly change the properties and quality of the finished food.

The lecithin commonly used is extracted from soybeans grown in the United States. There has been a steady increase in the soybean crop until now it exceeds 100,000,000 bu., and a steady decrease in the price of lecithin until now it is available at very reasonable cost for almost any industrial use. Purified hexane is used to extract the lecithin and oil from soybeans. During recovery the solvent is then distilled off from the extract, leaving a residue of lecithin suspended in oil. The lecithin is separated from the main body of the oil by hydration with steam and subsequent centrifuging. The lecithin emulsion as separated is then dried under vacuum at low temperatures.

This commercial product contains roughly equal proportions of lecithin, cephalin and soya oil, with small amounts of sterols and inositol. Sometimes the soya oil is removed by selective extraction with acetone, and replaced with another oil or a fat such as cocoa butter. This adsorbed oil or carrier serves to render the product indefinitely nonputrifying and readily dispersible.

Characteristics

Lecithin is a bright light-brown, soft, salve-like substance of neutral odor and bland taste. It can be bleached to a faint golden tint. At about 150 deg. F. it melts to an oil which disperses readily in warm glyceride oils. In the melted state it takes up limited percentages of alcohol, glycerol and other solvents to form combinations which remain fluid at room temperature. Commercial lecithin is only partly soluble in alcohol, but completely soluble in all aliphatic and aromatic hydrocarbons. With water it readily forms emulsions which can be diluted indefinitely.

Chemically, lecithin may be visual-

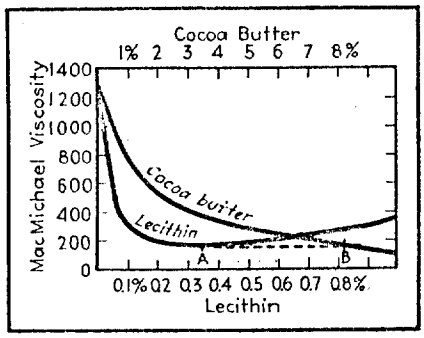

Fig. 1. Relative effect of lecithin and cocoa butter on viscosity of dark chocolate. (A) Most effective percentage of lecithin, 0.35 percent. (B) Percentage of cocoa butter, approximately 8 percent, required to give same viscosity as addition of 0.35 percent lecithin.

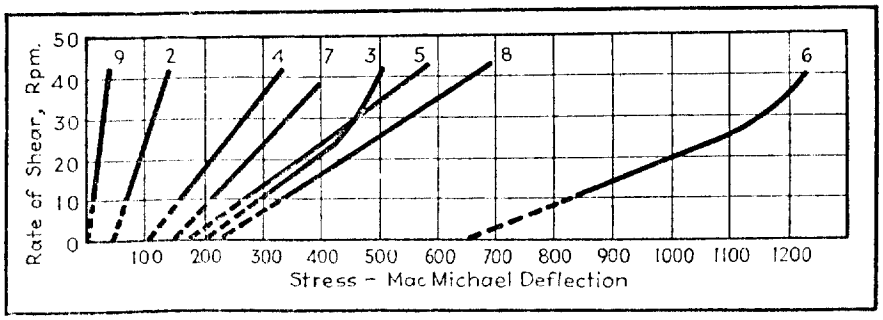

Fig. 2. Viscosity curves of typical chocolate products.[4] (2) Chocolate liquor. (3) Dark chocolate. (4) Light sweet chocolate. (5) Milk chocolate, 12 percent milk solids. (6) Milk chocolate, 18 percent milk solids, no lecithin. (7) Milk chocolate, 18 percent milk solids, with lecithin. (8) Buttermilk chocolate. (9) Ice cream coating.

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ized as a diglyceride, esterfied in turn to phosphoric acid and choline. The related and associated phosphatide, cephalin, has the same structure except that it contains ethanolamine in place of choline. The molecular structure of both lecithin and cephalin discloses a strongly lipophylic fatty acid group at one end of the molecule, and a strongly hydrophylic amine group at the opposite end. This naturally balanced antipolar configuration confers the interface properties by which a great many of its industrial applications are explained. When dispersed in any heterogeneous system, such as chocolate, margarine or ice cream, the phosphatides are visualized as forming a layer one molecule deep at the interface between the hetereogeneous constituents, with the fatty acid part of the molecules facing the lipophyle surface, and the amine part facing the hydrophyle surface. The practical result of adding a few tenths of a percent of lecithin to these foods appears magical. In the case of chocolate, a thick mud-like mass liquefies into a thin liquid almost instantly. With margarine, superior emulsion results in a product of increased utility for culinary purposes and for baking. Ice cream using high serum solids, in which lecithin effectively prevents crystallization of lactose, becomes a velvety smooth product, free of sandiness.

The antioxidant properties of lecithin are commonly ascribed to the amine and phosphoric acid parts of the molecule. Of course, lecithin has the obvious advantage of being a natural, innocuous and thoroughly nutritious antioxident for use in foods.

In itself, soya lecithin is a concentrated and valuable food occurring widely. The most important and vital organs and fluids of the vegetable and animal kingdoms contain lecithin or its associated phosphatides, and its presence adds to their dietetic value. Thus we find lecithin in meat, brains, nerves, blood and dairy products, as well as in all seeds, beans, nuts and grains.

If the lipids of foods containing only traces of oil are analyzed, they will be found to consist largely of lecithin and its associated phosphatides. In such low-fat foods we find the phosphatides generally complexed at interfaces with carbohydrates, proteins or compounds of silicon. Such a situation exists in flour, starch and skim milk, and in the foods manufactured from these. Let it not be assumed, however, that lecithin serves no function in these foods. For example, removal of the lecithin from gluten by ether extraction causes the gluten to lose its identity and degenerate into a useless crumbly mass. Its characteristic properties are restored by restoring the lecithin. For this reason one need not be surprised to learn that soya lecithin improves baked goods and macaroni. They depend largely on proper gluten functioning for quality.

Applications

When examining the interfacial and colloidal behavior of any system constituting a food, with a view of utilizing the advantages of adding soya lecithin, one can well bear in mind the chemical, physical, colloidal, antioxidant and physiological properties of soya lecithin as summarized.

Specific applications of soya lecithin in the food industries have been the subject of much study and many patent applications. In dispersions of finely divided material in a fat like chocolate, cocoa coatings, ice cream coatings[5] and wafer fillings, lecithin acts primarily as an edible wetting agent. By using lecithin, these products can be manufactured to the same fluidity with a much less oil or fat content. A flavorful undiluted food is thus produced with savings in labor, power, time and money. When lecithin serves as a wetting agent, most benefit is obtained when the interfaces or meeting points of the heterogeneous constituents are covered with an antipolar layer one molecule deep. Because of this fact the theoretical percentage of lecithin required is calculable from molecular constants. Depending on fineness, this optimal percentage generally figures out between 0.1 and 1 percent. This calculated percentage range checks with experience. This behavior shows another advantage of lecithin in that its benefits can be generally obtained by using only a few tenths of a percent in the finished food. Indeed, percentages beyond these low optimums may cause a reverse effect, as shown in Fig. 1.

Confectionery

In a heterogeneous system like chocolate, uniform wetting of the hydrophylic disperse phase results in colloidal stability. Recently the beneficial effect of lecithin in chocolate has been studied by mechanically measuring the plasticity or change in viscosity.[1] Cocoa butter, lecithin, moisture, air, milk solids, fineness, temperatures and processing have been shown to be among the factors influencing the plastic behavior of chocolate.

Besides stabilizing the chocolate, saving cocoa butter, and counteracting the thickening effect of moisture, lecithin protects colloidal dispersion, especially of milk chocolate, in the melted or overheated state (Table II). Viscosity curves also show how the lecithin counteracts gumminess and improves coverage of chocolate coating (Fig. 2). Chocolate stabilized with lecithin has been found to have a readily controllable temper when properly heat conditioned for enrobing. By widening the enrobing temperature range, and controlling undesirable crystallization of the higher melting glycerides with lecithin, production of chocolate goods of unusual stability, smoothness, luster and resistance to bloom at lower cost has been found possible.

Oil-containing confections like caramels and nougats are made essentially by boiling a sugar sirup with a fat. In such products, lecithin acts as a high-temperature emulsifying agent, causing rapid dispersion of the fat in the hot sirup. The result is a smooth, uniform, creamy confection. At the same time lecithin counteracts greasiness, graining and staling, to give a longer shelf life. This application of lecithin is im-

TABLE I—Effect of Heat on Viscosity of Chocolate Products as Measured by MacMichael Viscosimeter.

Sample No.	Kind	Viscosity before heating	Viscosity after heating
1	Cocoa butter	1	1
2	Chocolate liquor	95	90
3	Dark chocolate	370	357
4	Light sweet chocolate	224	220
5	Milk chocolate, 12% milk solids	554	489
6	Milk chocolate, 18% milk solids, no lecithin	1004	1830
7	Milk chocolate, 18% milk solids, with lecithin	268	364
8	Buttermilk chocolate	456	789
9	Ice cream coating, 55% cocoa butter	12	12
10	Ice cream coating, 65% cocoa butter	5	5

TABLE III—Effect of Moisture on Viscosity of Chocolate Products.

Moisture (percent)	With 0.3% lecithin	With 6% cocoa butter
0.50	112	112
0.75	130	132
1.00	143	150
1.25	187	195
1.50	240	238
1.75	250	290
2.00	330	440

TABLE II—Effect of Temperature on Viscosity of Chocolate

Sample No.	Kind	95° F.	100° F.	105° F.	110° F.	120° F.	130° F.	140° F.	150° F.	160° F.
2	Chocolate liquor	101	95	88	80	69	61	54	48	42
3	Dark chocolate	390	370	356	340	318	298	281	265	249
4	Light sweet	227	214	206	199	189	188	190	197	209
5	Milk chocolate, 12% milk solids	570	554	549	547	555	414	....	....	....
6	Milk chocolate, 18% milk solids, no lecithin	1015	1004	1004	1025	1100	1195	1335	1530	....
7	Milk chocolate, 18% milk solids, with lecithin	281	268	260	251	239	231	232	242	264
8	Buttermilk chocolate	463	438	413	392	359	344	340	355	390

portant to the extent that some manufacturers of oils and fats for the confectionery trade incorporate lecithin into all their products.

Ice Cream and Margarine

Soya lecithin has a special stabilizing effect in ice cream.[5] It imparts a velvety smoothness, as well as improving body, melt-down and heat-shock characteristics. Lecithin may also show striking advantages when used in conjunction with other stabilizers, such as glyceryl monostearate. Above all, it prevents crystallization of lactose in high-serum-solids ice cream, and in that way eliminates the problem of disagreeable sandiness. Low-serum-solids ice cream has a biting, frigid taste because of its low melting point. Lecithin-containing, high-serum-solids ice cream retains its smoothness with no sanding, even after storage and periodic heat-shocking for almost a year. This important use of soya lecithin furnishes a new avenue of disposition of fat-free milk solids at a reasonable price, which is important to our agricultural economy. This possibility is particularly important because of rising prices and shortages arising from war conditions.

Another essential application of soya lecithin is in the manufacture of margarine. In this industry lecithin completely displaced egg yolk many years ago because of its superior colloidal effects, stability against putrefication, and lower cost. Fractional percentages confer butter-like properties to margarine. A better intermixture of the various glycerides is also effected, producing a margarine of better appearance, texture and spreadability. In particular, lecithin-containing margarine does not spatter when used in frying, and gives a desirable brown, movable sediment which does not stick to the bottom of the pan. In addition, lecithin enhances the baking qualities of the fat, and owing to its antioxidant properties favorably affects the keeping qualities of margarine fortified with fish-liver-oil concentrates containing vitamins A and D.

Edible Fats and Macaroni

Since lecithin is a natural oil-soluble antixodiant[6] and emulsifier, it finds enthusiastic acceptance in edible fats and shortenings. As little as 0.05 percent can be used to counteract rancidity and hydrolysis in lard, oleo oil and compound shortening. During wartime its use in such fats is particularly recommended to stabilize them for long storage or export and to lessen other hazards. Higher percentages can be used in baking fats to increase their shortening and emulsifying effect in bread, cake and biscuit doughs.[7] The lecithin causes the shortening to spread rapidly and uniformly throughout the batch, promoting tenderness, moisture retention and even texture.

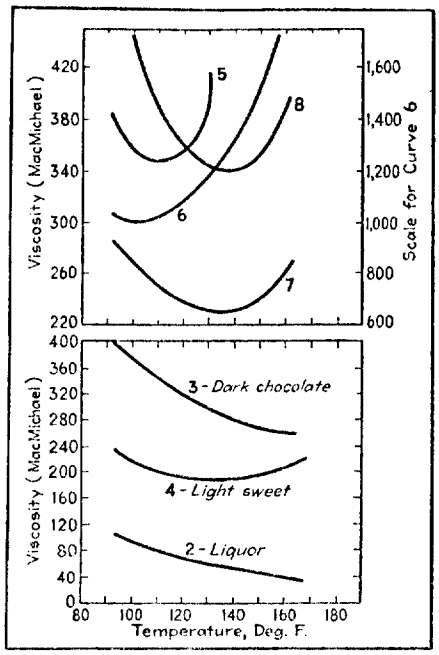

Fig. 3. Effect of temperature on viscosity of chocolate. Numbers on curves refer to Table II.

The advantages of soya lecithin in macaroni products are well known. A study of the gluten hydrogel formed on cooking macaroni reveals that lecithin is essential to its proper structure. The lecithin is adsorbed at the gluten interfaces (boundaries of the gluten particles), stabilizing the cooked macaroni to the extent of preventing syneresis or water separation.[8] Disintegration on prolonged cooking or canning is avoided by the use of soya lecithin.[9] The improving effect in macaroni is so great that the inclusion of 1 percent makes possible the manufacture from soft wheat flour of macaroni comparable in quality to that made from hard wheat flour.[10] In Germany, for example, where hard wheat for alimentary pastes must be imported, a decree of the government* legalizes soya lecithin in macaroni doughs in order to utilize domestically grown soft wheat.[11] Greater latitude in the selection of raw materials, especially the utilization of domestic raw materials, is of special importance during wartime.

Vitamin Fortification Problems

The role of lecithin in vitamin fortification is interesting. That soya lecithin stabilizes the potency of vitamin A preparations and concentrates because of its antioxidant effect has long been known.[12] Deficiency in choline causes derangements in fat metabolism giving rise to fatty livers. Choline is one of the few substances that furnish indispensable labile methyl groups.[13]

For these reasons Richardson[14] classifies choline among the accessory food factors of the vitamin B group. These accessory functions of choline are antagonized by cholesterol. Accordingly, soya lecithin is a good source of choline, because, unlike eggs, it contains no cholesterol. The naturally esterified choline in soya lecithin shows none of the undesirable after effects of synthetic choline. That increased metabolism or increased ingestion of thiamin requires additional choline has been shown.[15]

Unfortunately the over-refining of fats, sugars and flour has depleted our food of vitally necessary choline, as well as vitamins and minerals. Hence, fortification of food with thiamin should be accompanied by the addition of soya lecithin as well, to provide the cholesterol-free choline which makes the vitamin more effective.

Soya lecithin has heretofore been looked upon as a specialty ingredient. But because of the properties and proved applications of lecithin, and because of its availability, soya lecithin justifiably can be regarded as a commodity which may assume first-rank importance in the conservation of, and more effective use of, strategic materials such as fats and vitamins.

* D. R., Entwurf einer Verordnung über Teigwaren, Secs. 2 & 5.

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A SEMI-MICROMETHOD FOR PHOSPHOLIPID DETERMINATION

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(Received July 23, 1971)

SUMMARY

1. A semi-micromethod for phospholipid determination is described based on the principle of phospholipid determination used by Baumann for extraction of total lipids and using the method of Fiske and Subarrow for phosphate determination.

2. The method requires less time and is performed at minimal cost on a small amount of serum.

THE ESTIMATION OF SERUM PHOSPHOLIPID IS BEING USED INCREASINGLY IN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES. In normal fasting subjects, phospholipids constitute the largest fraction of serum lipids; the distribution averages 60-70 per cent phosphatidyl choline, 15-20 per cent sphingomyelin, 5 to 8 per cent phosphatidyl ethanolamine and phosphatidyl serine and the remainder lysophosphatidyl cholines, plasmalogen and other less well characterized material. Phosphatidyl choline and sphingomyelin act as mild detergents contributing to the stabilizing effect of phospholipid in the lipid transport system.

Because phosphatide may be measured for investigation of fat metabolism, it is essential to have a reliable, practical method for its determination. Most of the methods in current use involve the oxidation of the phosphatides contained in a purified lipid extract followed by measurement of the inorganic phosphorus liberated. Virtually all of the analytical methods depend on the formation of phosphomolybdate ion, which is then reduced by a suitable reagent to form the complex "heteropolyblue" a colloidal dispersion with a composition not precisely known.

The present method is a modification of the method of Baumann *(1)* for extraction and Fiske and Subarrow *(2)* for inorganic phosphorus determination.

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Material and Method

Blood samples were taken from twenty subjects following a 14-16 hr fast for comparison of the semi-micromethod with macro-p-semidine method *(3)*. Duplicate analysis of samples were done on the same day for each method. Using the semi-micromethod 0.1 ml of serum was added to 5.0 ml of a 3:1 (V/V) mixture of ethanol and ether in a glass stoppered centrifuge tube. This was allowed to stand for ten minutes and then centrifuged at 2000 rpm. Two ml of the supernatant was transferred to another tube and evaporated to dryness. To the residue, standard and blank, 2.0 ml of 1.5 normal sulfuric acid and a few drops of hydrogen peroxide were added and heated. Additional drops of hydrogen peroxide may be required for the blackish residue to turn colorless. 1.0 ml of 2.5% ammonium molybdate solution and 1.0 ml of 1-amino-2-napthol-4-sulfonic acid were added for color development. After thirty minutes the unknown together with a standard containing 0.04 ml of 10 mgs% phosphorus solution was read against a reagent blank with the galvanometer light of the spectrophotometer set at 100 per cent transmittance or zero absorbancy and wavelength 800 nanometers. A commercial control serum was treated in the same way as the unknown. The phospholipid is expressed as lecithin.

Results

Table 1 shows a comparison of twenty phospholipid values done by the semi-micromethod and the P-semidine method. Individual values are similar for each method and using the paired Student "t" test, the calculated "t" value is not significant when $P = 0.9$.

Table 2 shows results of ten determinations by each method using mean value of the duplicate pooled serum. The standard deviation was 4 mg/100 and the coefficient of variation 1.5% for the semi-micromethod. This confirms the accuracy of the method.

Discussion

Several methods for the determination of phospholipids are in current use. In common with these established methods, in the semi-micromethod, phospholipid in the serum is first extracted, digested and the inorganic phosphorus is determined in the residue. *(1-4)* Contrary to claims that single extraction of lipids is incomplete and contaminated by non-phospholipid phosphorus, *(5-7)* Van Slyke proved this was not so when Bloor's reagent is used for extraction *(8)*. In the semi-micromethod the serum is added directly to the solvent and causes fine dispersion of the protein precipitate. When allowed to stand for ten minutes or more complete extraction occurs.

TABLE 1

COMPARISON OF PHOSPHOLIPID VALUES BY SEMI-MICRO AND P-SEMIDINE METHOD (mg/100)

Patient	Semi-Micro	P-Semidine
M.M.	213	213
M.M.	183	180
M.M.	210	208
M.M.	225	225
C.D.	255	253
M.M.	253	253
M.M.	253	248
C.L.	270	278
C.L.	240	230
S.P.	295	283
S.P.	275	268
C.D.	313	310
H.P.	560	570
C.J.	478	490
W.Y.	360	370
P.E.	283	283
P.J.	300	313
P.B.	258	258
P.B.	270	258
P.B.	268	268
	$\bar{X}$ 268	287
		t test not significant

TABLE 2

COMPARISON OF PHOSPHOLIPID VALUES BY SEMI-MICRO AND P-SEMIDINE METHOD USING POOLED SERUM (mg/100)

	Semi-Micro	P-Semidine
	263	270
	265	265
	260	268
	263	268
	270	265
	260	263
	268	270
	268	268
	265	268
	273	270
$\bar{X}$	266	268
S.D.	8(+2 S.D.)	6(+S.D.)
C.V.	1.5	1.1
		t test not significant

Hydrogen peroxide used during wet digestion interferes with colour development if not entirely removed. Its removal could be accomplished either by heating or by adding two drops of 5% urea solution after digestion *(9)*.

The advantage of this method is that only a small amount of serum is required, minimum equipment is used and the time involved is considably shorter than for other methods. It can therefore, be readily used in a routine laboratory as well as for research purposes.

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Table 1. Changes in the chemical shift of benzylic and aromatic protons of DDT (0.009 molal) by the addition of lecithin in CCl_4 at 33°C.

Lecithin concentration (molal)	Chemical shift (hz)		
	Benzylic proton	Ring proton b	Ring proton c
0	495.2	746.0	726.3
0.072	497.6	747.3	726.4
.123	499.2	747.6	726.5
.156	500.1	747.9	726.5
.17	501.8	748.3	726.9
.25	502.6	748.9	726.7
.293	503.9	749.3	726.7
.332	504.7	749.6	726.9
.368	506.2	749.9	726.7

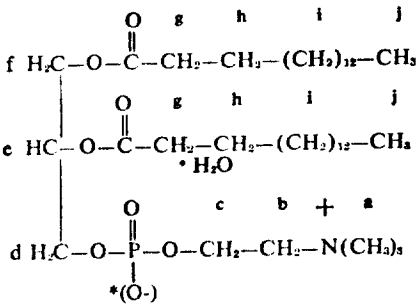
be obtained if solutions of DDT stand at room temperature (*2*). In this instance the spectral changes have been attributed to intermolecular charge-transfer complexing of the DDT. Wilson and coworkers (*2*) have shown, however, that DDT can act as a relatively weak electron donor in forming a charge-transfer complex with tetracyanoethylene. Changes in the chemical shift of the benzylic proton produced by aromatic donors have also been attributed to charge-transfer complexing. In this instance the DDT would be presumed to be acting as an acceptor (*3*).

In another way, Hilton and O'Brien (*4*) have shown that DDT will block the action of valinomycin on a phospholipid bilayer. Addition of this cyclic antibiotic to a lecithin-decane bilayer produces an increase in conductance across the bilayer, and this response is blocked by the subsequent addition of DDT. In this respect DDT differed from lindane and dieldrin. Whether the DDT interacted with the valinomycin, the lipid, or both constituents is not yet apparent.

To define the molecular basis for the action of DDT will require further information on the nature of its interaction with the component molecules of affected systems. One obvious omission at present is the lack of data concerning the interaction of DDT with complex lipids. We now discuss the interaction of lecithin and DDT as it is defined by nuclear magnetic resonance spectroscopy.

Proton magnetic resonance (PMR) spectra of lecithin and DDT in $CHCl_3$ or CCl_4 were recorded on a Varian HA 100-Mhz spectrometer, with tetramethylsilane (TMS) as an internal lock standard. The chemical shifts were determined with a precision of ± 0.25 hz. The β,γ-dipalmitoyl-DL-phosphatidylcholine monohydrate (Sigma) was used in these experiments as obtained, without further purification. Reagent grade DDT (City Chemical) and spectrograde chloroform and CCl_4 were used.

The PMR spectrum of this particular lecithin has been described (*5*). As was expected from the structure of this compound (**1**), the spectrum gave peaks with varying degrees of multiplicity which corresponded to protons a to i. In addition, the PMR spectrum always showed a peak whose chemical shift was dependent on the concentration, the



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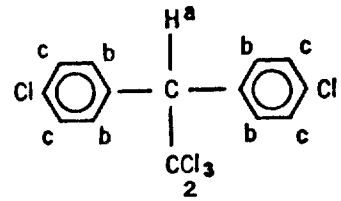
temperature, and the solvent. This peak was attributed to a condensed water molecule associated with the lecithin. Addition of DDT (0.770 molal) to a lecithin solution (0.045 molal) in $CHCl_3$ produced low field changes in the chemical shift of various lecithin protons. The $-N(CH_3)_3$ protons peak showed the maximum change in chemical shift (~11 hz), whereas proton b was shifted 5 hz. Other lecithin protons also showed very small low field changes in chemical shift (~2 hz). However, the position of the terminal methyl proton j was unaffected. The associated water proton peak also gave a low field shift.

The PMR spectrum of DDT (**2**) in $CHCl_3$ or CCl_4 showed a sharp peak due to the benzylic proton "a" at approximately 5.0 parts per million (*6*). The ring proton



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spectrum showed a complex pattern because of ortho and meta spin splitting between the magnetically nonequivalent ring protons (*6*). Addition of the lecithin to a solution of DDT produced low field changes in the chemical shift of the benzylic proton.

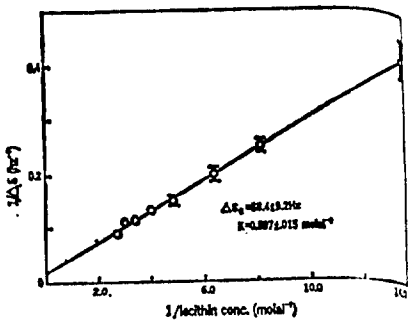

Fig. 1. Reciprocal plot of change of chemical shift of benzylic proton of DDT as function of lecithin concentration at 33°C.

The ring protons "b" showed similar though much smaller changes in chemical shift. No appreciable change in the chemical shift of the ring protons "c" was observed (Table 1).

These induced changes in chemical shift would suggest an interaction between lecithin and DDT. Apparently the rate of proton exchange between the lecithin-DDT complex and the reactants is very rapid on the nuclear magnetic resonance time scale, and only one average peak is observable.

The equilibrium for the binding of DDT to lecithin can be expressed as

$$(\text{Lecithin}) + (\text{DDT}) \rightleftharpoons (\text{Lecithin}) \cdot (\text{DDT}) \quad (1)$$

The equilibrium constant K can be calculated with the use of the following expression (*7*)

$$\frac{1}{\Delta\delta_o} = \frac{1}{K\Delta\delta_c C} + \frac{1}{\Delta\delta_c} \quad (2)$$

where C is the concentration of lecithin and δ is the chemical shift in parts per million. $\Delta\delta_o$ is the difference between δ(observed) and δ(noncomplexed DDT) and $\Delta\delta_c$ is the difference between δ(complexed DDT) and δ(noncomplexed DDT). The above relation (Eq. 2) is valid only when the concentration of DDT is much less than that of the lecithin. If $1/\Delta\delta_o$ is plotted as a function of $1/C$ (Fig. 1) and a least squares fit is used, $\Delta\delta_c$ is calculated to be 58.4 ± 9.2 hz and K to be 0.597 ± 0.015 molal^{-1}.

The inductive effect of the three chlorine atoms would increase the acidic character of the benzylic proton which, consequently, would be more likely to associate with an electronegative atom such as the oxygen bound to the phosphorus. The change in electronic environment produced by such an interaction could account for the observed changes in the chemical shift of the benzylic proton and the ring protons,

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Binding of DDT to Lecithin

Abstract. *An interaction between DDT and lecithin is indicated by the reciprocal effects of each compound on the proton magnetic resonance spectrum of the other. The phosphoryl choline moiety of the lecithin and the benzylic proton of the DDT seem to be involved. The most pronounced response in the proton magnetic resonance spectrum of the lecithin produced by increasing concentrations of DDT was a change in the chemical shift of the resonance peak due to the protons of the choline methyl groups. Increasing concentrations of lecithin produced changes in the chemical shift of the resonance peaks of the benzylic proton and adjacent ring protons of the DDT. Equilibrium constant of 0.597 ± 0.015 molal^{-1} was obtained for this interaction.*

The toxicity of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] is generally attributed to its effect on the central nervous system where it produces an excitatory effect on axons. In an effort to define the molecular basis of the response, Matsumura and O'Brien have demonstrated that DDT will bind to components of cockroach nerve (*1*). Using spectral and fluorescence data these workers have postulated the formation of a charge-transfer complex. This hypothesis is open to criticism because comparable spectral changes can

The ring protons, c, are further removed from the site of interaction and would not be expected to respond to the same degree.

Transmitted changes in the electronic environment resulting from this type of interaction would account for the changes in chemical shift of the lecithin protons. The more pronounced changes in the chemical shift of the *N*-methyl protons could result from their proximity to the interacting molecule. Normally one would expect the phosphorylcholine moiety to assume a conformation allowing interaction of the quaternary nitrogen and the negative charge of the phosphate.

The effect of the DDT on the resonance peak of the protons of the associated water molecule depends on the initial concentration of the lecithin. At concentrations higher than 0.018 molal of lecithin, a low-field change in the chemical shift of lecithin is observed by the addition of DDT. In more dilute solutions of lecithin, however, DDT produced significant line broadening with only small changes in chemical shift.

The potential for such an interaction in biological systems will depend on the nature of the environment of the lecithin molecule in a membrane, and this situation has not yet been defined. However, a rather strong association can occur between lecithin and DDT, and further studies in more complex systems should provide some indication of the significance of this interaction. The involvement of the benzylic proton of the DDT is of significance, and this observation would substantiate observations of Ross and Biros (*3*).

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DEPARTMENT OF SURGERY
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July 2, 1974

Humphrey F. Sassoon, PhD.
Tracor Gitco Inc.
1300 East Gude Drive
Rockville, Maryland 20851

Dear Dr. Sassoon:

Thank you for your letter of June 26th requesting information on our studies with lecithin. The citation which you inquired about published as an abstract in Clinical Research, Volume 21, page 276, 1973 is a part of a larger study which we are now in the process of compiling for manuscript production. We are waiting final chemical analyses prior to publication of this and when it appears will contain all the data which was reported as the abstract which you saw.

I am taking the liberty of enclosing a recent publication of ours dealing with lecithin which you may find interesting from your standpoint. This article references the clinical research abstract as well. Thank you for your interest in our work.

Sincerely Yours,

R. K. Tompkins

Ronald K. Tompkins, M.D.
Associate Professor of Surgery

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A Discoursive Review of the Present Knowledge of Lecithin and Related Substances

Compounds of "greasy or lipid materials" containing phosphorus as phosphoric acid have been recognized as constituents of living cells of both animals and plants for many years.

In 1719 Hensing demonstrated that phosphorus is a normal constituent of brain.

Vauquelin in 1812 isolated fatty materials containing phosphorus from brain.

Gobley (1846, 1847) isolated a phosphatide (phospholipid) from egg yolk which he called lecithin (Greek lekithos = egg yolk).

Couerbe, Fremy, and Valencienne (1834-1857) found phosphatides in a variety of animal sources, and in 1861 Topler found them in plant seeds. Thus, by the middle of the last century the widespread occurrence of phosphorus-containing lipids in tissue and tissue products was established.

The composition of Gobley's crude egg yolk lecithin had been pretty well worked out by 1868 (Diakanow and Strecker), and shown to contain fatty acids, glycerol, phosphoric acid, and the base, choline.

A type of phosphatide differing from lecithin was isolated from brain by Thudichum in 1884 and called kephalin (from Greek kephale meaning head) but now spelled cephalin. This material, like the early lecithin preparations was impure, being a complex mixture of phosphatides. Many workers throughout succeeding years worked at trying to separate lecithin and cephalin from these mixtures and establish their chemical composition. By 1915 it was shown that the main phosphatide fraction of brain called cephalin is made up of fatty acids, glycerol, phosphoric acid and the base, ethanolamine or aminoethyl alcohol. It appeared that these components in cephalin were linked together as in lecithin, with ethanolamine taking the place of choline.

So-called cephalin was found, like lecithin, to be a widespread constituent of tissues and associated with lecithin.

Since it was obvious that the so-called cephalin fraction of brain was a mixture of substances differing from lecithin, various workers set about trying to separate other components. Finally Folch (1941,1942) isolated a pure substance which contained the amino acid L-serine in place of ethanolamine.

Prior to 1940 the substance referred to as cephalin was the derivative containing ethanolamine, but after Folch's discovery of the substance containing serine in the crude cephalin fraction the inadequacy of nomenclature became obvious.

The complexity of the cephalin problem grew with the discovery by Klenk and Sakai (1939) of the cyclic polyhydroxy compound inositol in the so-called cephalin fraction of soy beans. Folch and Woolley (1942) then found inositol to be a component of the phosphatides of brain and spinal cord. Folch (1946,1949) purified his material and showed inositol to be present as the metadiphosphate. He called the substance "diphosphoinositide". When hydrolyzed the substance was broken up into fatty acids, glycerol, and inositol metaphosphate.

Woolley (1943) showed that soy beans are a rich source of inositol-containing phosphatides, and this material he called "lipositol". The material undoubtedly was not a pure substance, and was found to be a very complex material, yielding upon hydrolysis ethanolamine, tartaric acid, galactose, fatty acids and inositol monophosphates.

Hawthorne and Chargaff (1954) found both inositol monophosphate and diphosphate in the hydrolysis products of the inositol phosphatide fraction of soy beans.

Folch isolated a purer preparation of inositol phosphatide from crude soy bean phosphatide which he called soy bean monophosphoinositide. This material was made up of unidentified primary amine, 1 mol; galactose, 2 mols; glycerol, 2 mols; inositol, 2 mols; fatty acids, 3 mols; and phosphoric acid, 2 mols. The relation of this substance to Woolley's "lipositol" is uncertain.

Inositol-containing phosphatides have been found in cottonseed and liver, and undoubtedly are rather widely distributed in plants and animals.

Thudicum, in his classical investigation of the composition of the brain (1901) obtained a phosphatide from brain which he called sphingomyelin, and which was later shown to be composed of the complex amino alcohol, sphingosine, phosphoric acid, choline, and fatty acid.

Sphingomyelins have been found in brain, spinal cord, kidney, liver, blood, egg yolk, muscle and other organs, in association with other phosphatides. They apparently have not been found in vegetable sources.

Classification of the phosphatides (phospholipids)

Until a few years ago, before the complexity of the so-called cephalin fraction of phosphatides was recognized, their classification was simple:

Lecithins

Cephalins

Sphingomyelins

At present, as the result of the discovery of different types of substances in the so-called cephalin fraction, the classification has been revised to take this into account. While different classifications have been proposed, the following scheme of Folch (Phosphorus Metabolism, Vol. II, page 187, 1952. The Johns Hopkins Press, Baltimore) appears most satisfactory.

I. The phosphoglycerides. Phosphatides or phospholipids in which the alcohol residue is glycerol.

(a) Phosphatidyl cholines -- lecithins

(b) Phosphatidyl ethanolamines
(c) Phosphatidyl serines
} -- cephalins

(d) Phosphatidic acids

(e) Acetal phosphatides

(f) Cardiolipins

II. Phosphosphingosides. Phosphatides or phospholipids in which the alcohol residue is the complex amino alcohol sphingosine.

(a) Sphingomyelins

III. Phosphoinositides. Phosphatides or phospholipids in which the alcohol residue is the cyclic alcohol inositol.

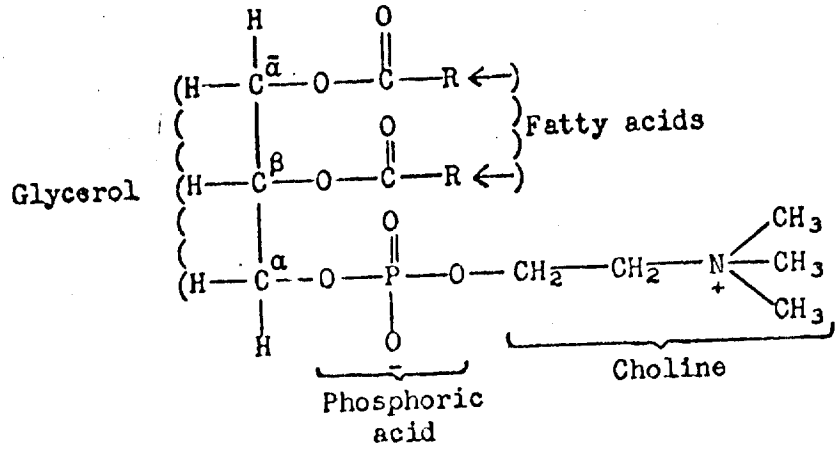
(a) Lipositol of soy beans)
(b) Monophosphoinositide of soy beans) -- cephalins
(c) Diphosphoinositide of brain)

It will be noted that what formerly was called "cephalin" really is a profound mixture of phosphoglycerides (phosphatidyl ethanolamine and phosphatidyl serine) and phosphoinositides.

Since commercial soy bean "lecithin" contains roughly 29 per cent true lecithins, 29 per cent phosphatidyl ethanolamine and possibly a little phosphatidyl serine, and about 31 per cent of the complex phosphoinositides (lipositol, monophosphoinositide) it represents a tremendously complex group of substances. Also, since as yet the composition of soy bean phosphatides is imperfectly known and may in the future show the presence of other phosphatide substances, an outline of the specific chemical compositions of phosphatides according to the above classification may be helpful as background for later discussion. These will be considered in classification order. This material may be found in the book "The Phosphatides", by Wittcoff. Reinhold Publishing Corporation, New York, 1951; and in the review of Folch in "Phosphorus Metabolism", Vol. II, page 186. The Johns Hopkins Press, Baltimore, 1952.

I. The phosphoglycerides

(a) The phosphatidyl cholines or the lecithins. These substances are composed of fatty acids, glycerol, choline, and phosphoric acid of the general chemical structure.



Different lecithins vary through variation in the kind of fatty acid groups present. Often both a saturated and an unsaturated acid are present, though lecithins with only saturated or unsaturated acids have been found. Even carbon unsaturated fatty acids from C_{16} to C_{24} have been found. The fatty acids, palmitic (C_{16}) and stearic (C_{18}) are the chief saturated acids present. The acids present in soy bean lecithin are (Thornton, Johnson and Ewan, 1944):

Acid	Per cent
Palmitic, C_{16}	15.77
Stearic, C_{18}	6.30
Oleic, C_{18}	12.98
Linoleic, C_{18}	62.92
Linolenic, C_{18}	2.02

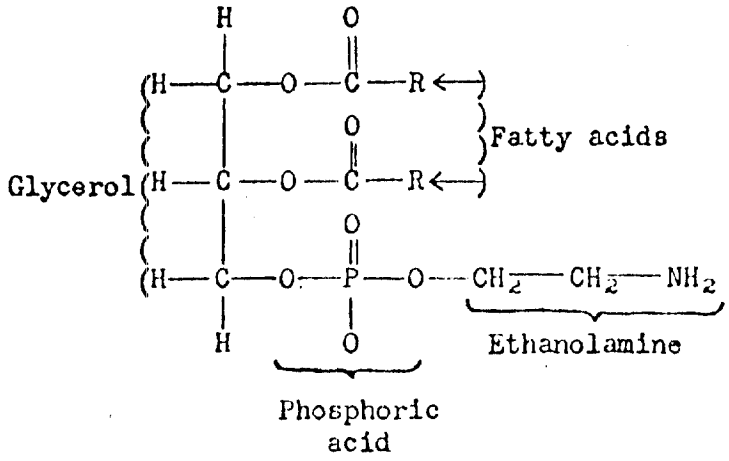
Each per cent represents the per cent of the total fatty acids present.

The whole phosphatide fraction of soy beans, in addition to the above acids, has been found to contain appreciable amounts of an unsaturated hexadecenoic acid (C_{16}) and a small amount of saturated arachidic acid (C_{20}).

The large amount of the "essential" linoleic acid in soy bean phosphatides is of importance.

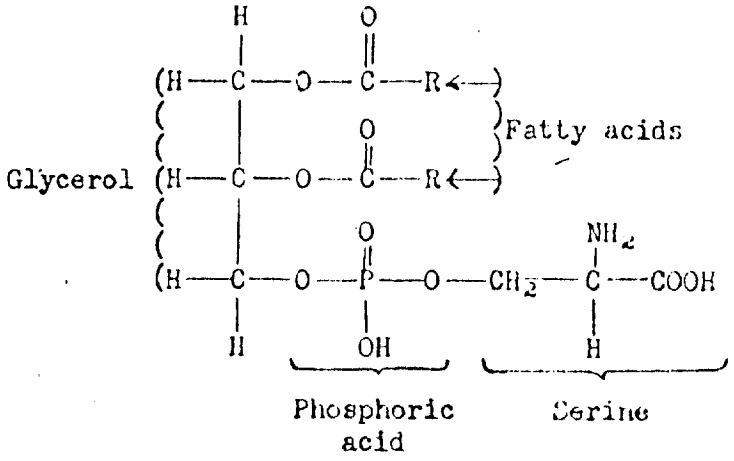
(b) The phosphatidyl ethanolamines. These compounds are composed of fatty acids, glycerol, ethanolamine, and phosphoric acid, and in structure are

analogous to the phosphatidyl cholines or lecithins:

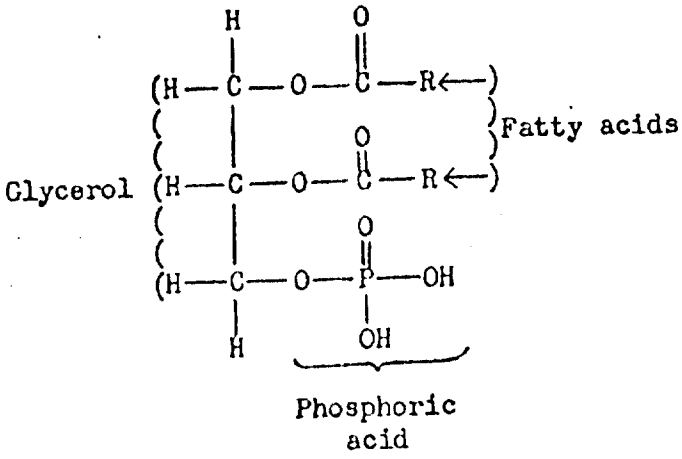


The phosphatidyl ethanolamines are differentiated by differences in the fatty acid components just as are the phosphatidyl cholines or lecithins.

(c) Phosphatidyl serines. These phosphatides have structures analogous to those of phosphatidyl cholines and phosphatidyl ethanolamines except that the amino acid serine takes the place of choline or ethanolamine:

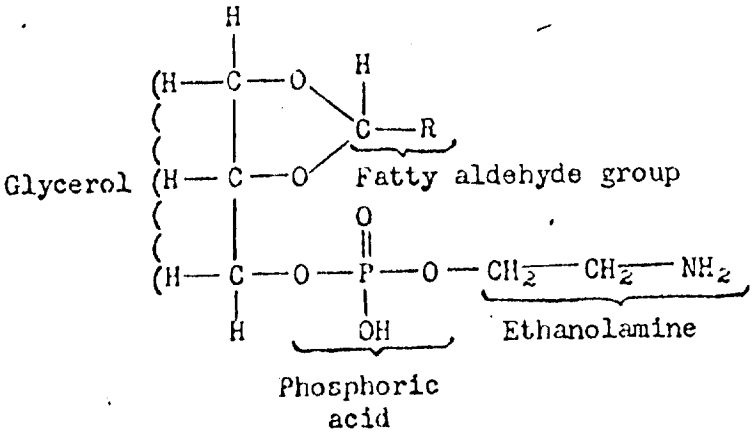


(d) Phosphatidic acids. These substances are composed of glycerol, fatty acids, and phosphoric acid:



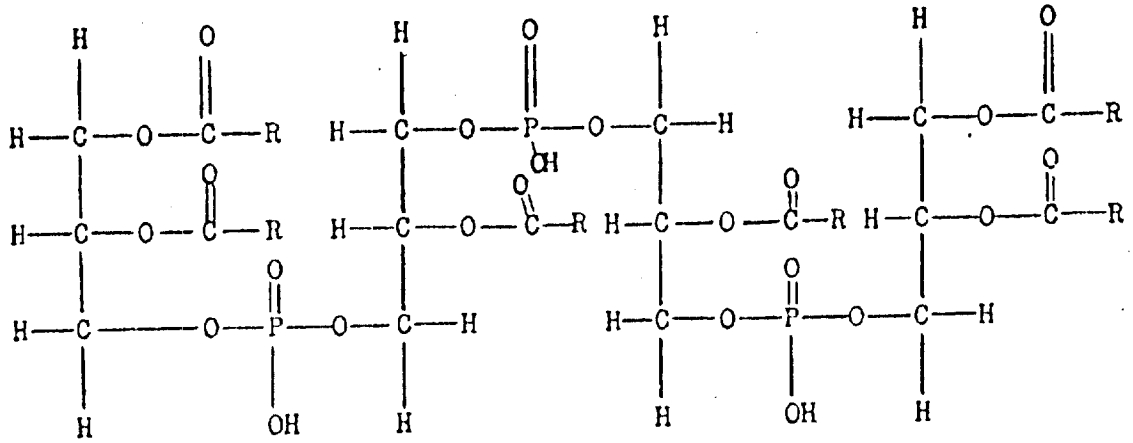
The phosphatidic acids are formed from glycerophosphatides a, b, c above by splitting off choline, ethanolamine or serine respectively. They have been isolated from cabbage leaves and carrots. As shown later they appear to represent intermediate products in the synthesis of glycerophosphatides in the animal body.

(e) Acetal phosphatides. These phosphatides are composed of glycerol, fatty acids, ethanolamine, phosphoric acid, and a higher fatty aldehyde such as stearic aldehyde or palmitic aldehyde. They have the structure:



The acetal phosphatides occur widespread in animal tissues, and are especially abundant in muscle and brain. They have not been found in plants. Nothing is known of their function.

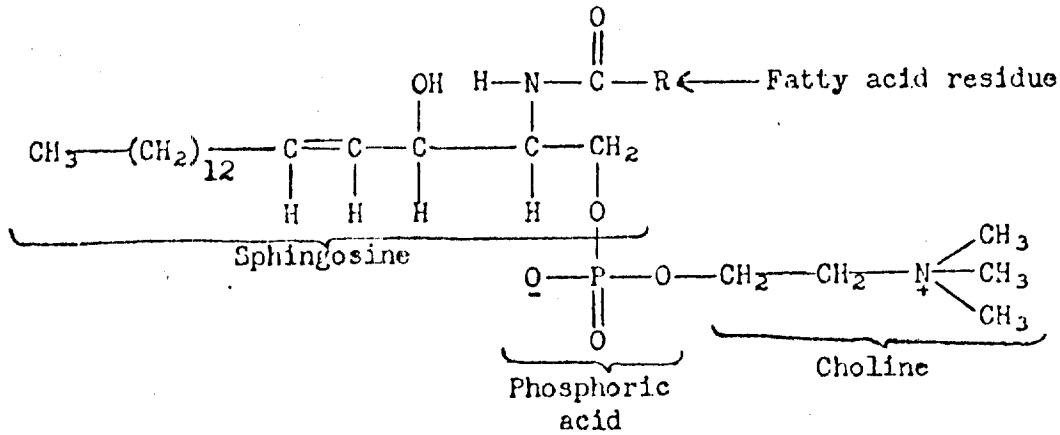
(f) Cardiolipins. These phosphatides are complex substances made up of glycerol, phosphoric acid, and fatty acids, and appear to have the structure:



The R—C— groups represent fatty acid residues attached to glycerol residues. The structure represents a fatty acid derivative of polyglycerophosphoric acid. Cardiolipin was isolated from heart, and there is some evidence for such compounds in egg yolk and liver phosphatides. Nothing is known as to its importance except that it appears to be involved in beef heart extract used in carrying out the ...rological test for syphilis.

II. Sphingomyelins

These substances are made up of the amino alcohol sphingosine, fatty acid, choline and phosphoric acid:

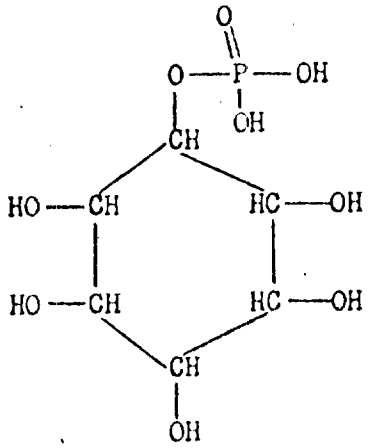


Sphingomyelins are differentiated by containing different fatty acids. They occur widespread in animal tissues and blood, but apparently not in plants. They are especially abundant in brain and spinal cord and undoubtedly are essential

components. Function unknown.

III. Phosphoinositides

(a) Lipositol. This phosphatide of Woolley probably does not represent a pure substance. Its complexity is indicated by the fact that upon hydrolysis it yields galactose, fatty acids, tartaric acid, ethanolamine, and inositol monophosphate.



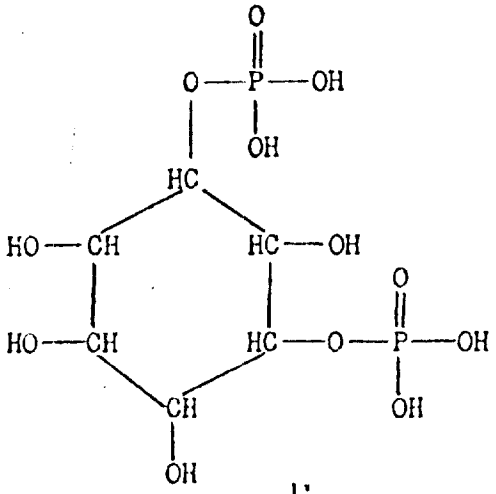
Inositol monophosphate

Nothing more is known about the structure of this substance.

(b) Monophosphoinositide of soy beans. Folch showed this substance to consist of an unidentified primary amine, 1 mol; galactose, 2 mols; glycerol, 2 mols; inositol, 2 mols; fatty acids, 3 mols; and phosphoric acid, 2 mols.

Hawthorne and Chargaff (J. Biol. Chem., 206, 27, 1954) demonstrated the presence of inositol monophosphate (structure above) in the inositol phosphatides of both soy beans and ox brain. These workers also obtained substances which were thought to be arabinose and galactose compounds of inositol monophosphate. Scholfield and Dutton (J. Biol. Chem., 208, 461, 1954) demonstrated that the inositol phosphatides of soy beans can be separated into two inositol-containing fractions, one of which contains the nitrogen base ethanolamine and the other is nitrogen free.

(c) Diphosphoinositide of brain. Folch has shown this substance to be composed of glycerol, fatty acids, and inositol meta-diphosphate in equimolecular proportions.



Inositol metaphosphate

Nothing more is known about the structure of this substance.

It will be seen from the above discussion that the complex phosphatides of soy beans contain most of the chemical units found in the chief phosphatides of animal tissues.

These are represented by:

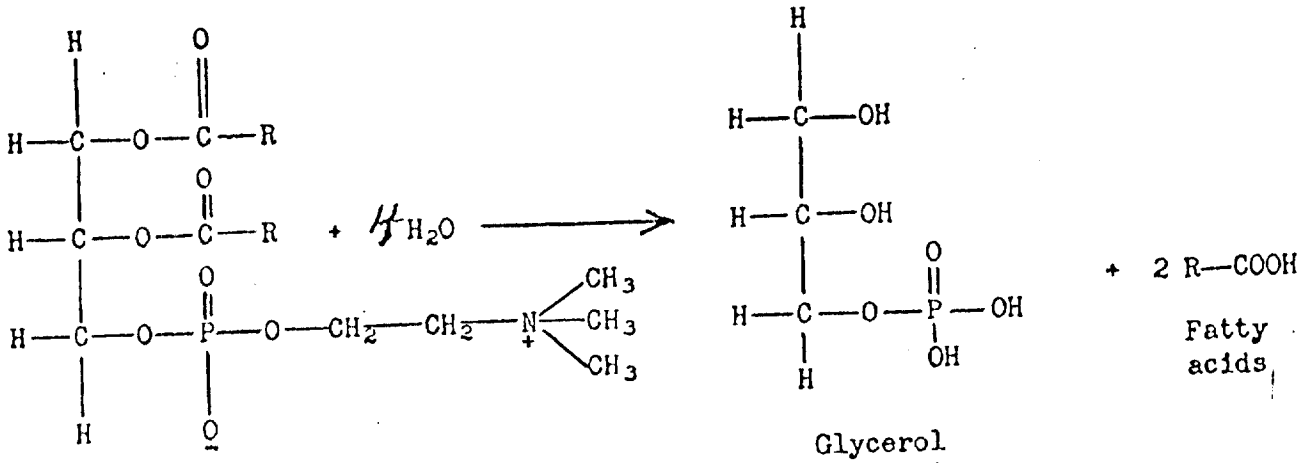
I. Phosphoglycerides:

(a) Phosphatidyl cholines - lecithins	about 29%
(b) Phosphatidyl ethanolamines	29%
(c) Phosphatidyl serines (small amount)	
(d) Phosphoinositides, or inositol-containing phosphatides	31%

It is interesting that the three groups of substances are present in roughly equal percentages in soy bean phosphatide.

Digestion of the phosphatides or phospholipids

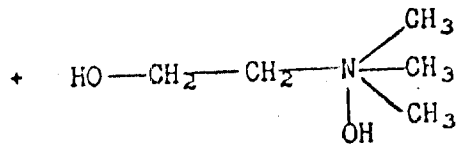
Apparently Bokay in 1877 first observed the enzymatic digestion of lecithin when he found the action of pancreatic juice to split it into glycerol phosphoric acid, fatty acids, and choline.



Lecithin

Glycerol Phosphoric acid

+ 2 R—COOH Fatty acids



Choline

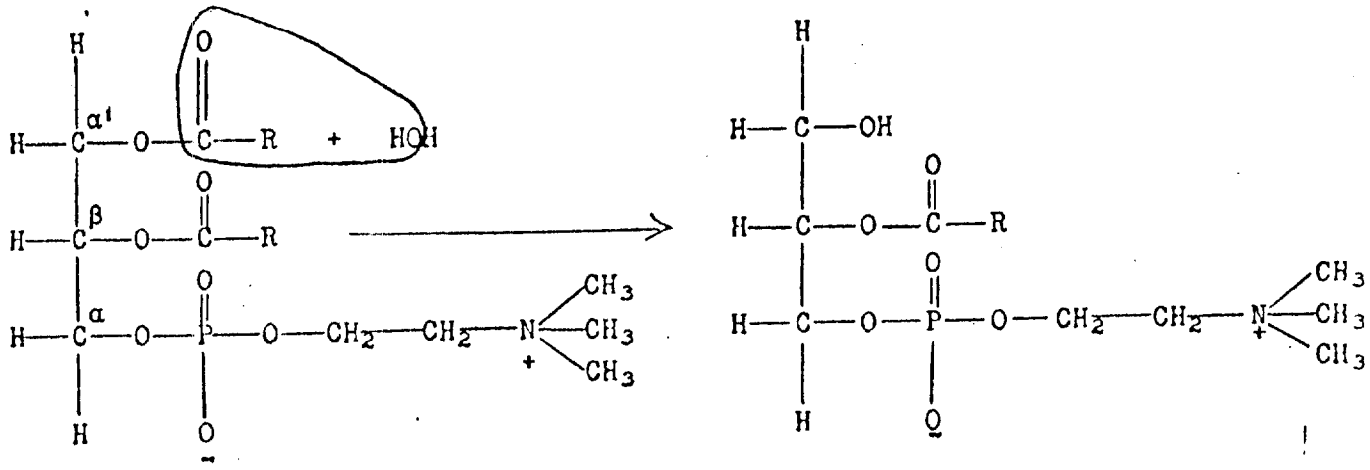
This observation was confirmed by Mayer in 1906.

Only the enzymes which hydrolyze the phosphoglycerides in the sub-groups hosphatidyl cholines (lecithins) and phosphatidyl ethanolamines have been worked on ppreciably.

It appears that the same enzymes which hydrolyze the lecithins (phosphatidyl holines) also hydrolyze the phosphatidyl ethanolamines. Presumably these enzymes r others also hydrolyze the phosphatidyl serines. Essentially nothing is known bout the enzymatic hydrolysis (digestion) of sphingomyelins or the inositol-containing hosphatides (phosphoinosides).

The enzymes which hydrolyze the lecithins and other phosphatides indicated above re called "lecithinases" and four types are known according to the points of ydrolysis. These lecithinases are referred to as lecithinases A, B, C, and D.

I. Lecithinase A. This enzyme splits off one fatty acid from the molecule nd forms a lysolecithin, so-called because it hemolyzes red cells.



Lecithin

Lysolecithin

+ R—COOH

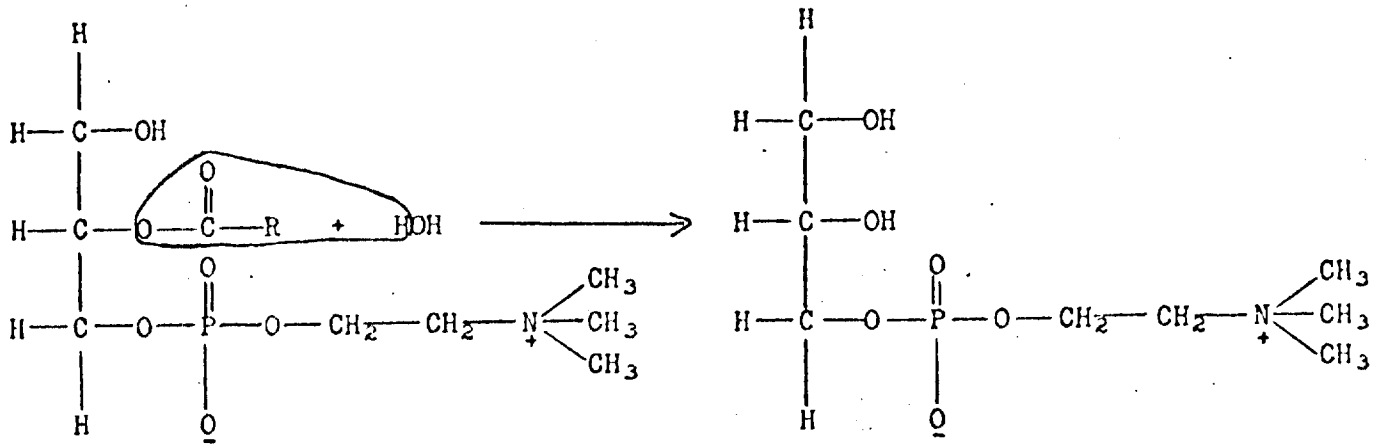
Fatty acid

Formerly it has been considered that lecithinase A hydrolyzes off only a olecule of unsaturated fatty acid. Hanahan (J. Biol. Chem., 207, 879, 1954) s shown the position of hydrolysis to be the fatty acid residue attached to he α' carbon as shown above, regardless of whether the fatty acid is saturated r unsaturated, though apparently the fatty acid in the α' position is generally nsaturated.

Lecithinase A is found especially in the venoms of poisonous snakes (cobra etc.), n pancreatic juice, and in various animal tissues. In the fresh organs the action f lecithinase A is held in check by other enzymes or substances. Upon drying the issues lecithinase A becomes active.

II. Lecithinase B. This enzyme removes the remaining fatty acid from the ysolecithin formed by the action of lecithinase A and yields α-glycerylphosphoryl- holine:

2963



Lysolecithin

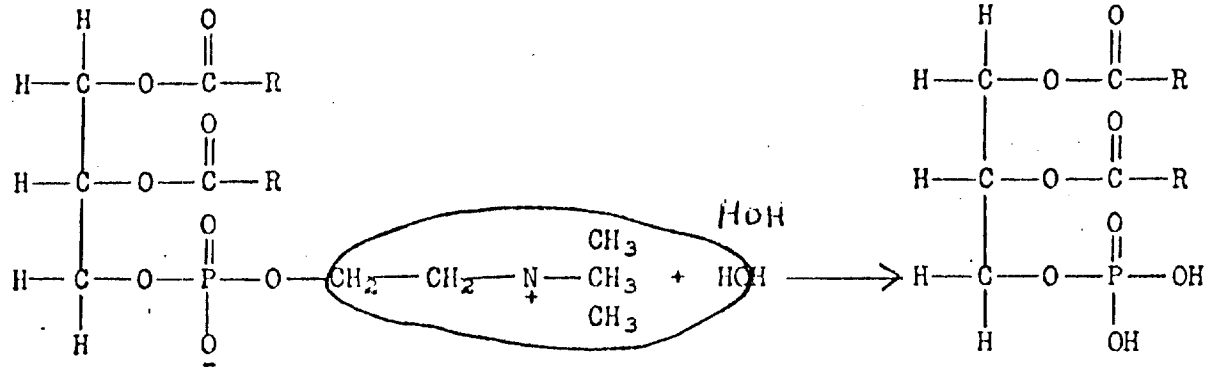
α-Glycerylphosphorylcholine

+ R—COOH

Fatty acid

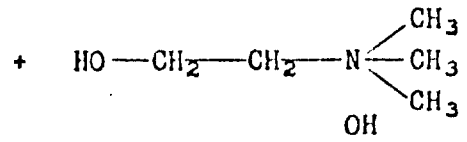
Lecithinase B occurs in pancreatic juice, pancreas and other animal tissue nd in higher fungi.

III. Lecithinase C. This enzyme splits choline from lecithins to form osphatidic acids. It also splits choline from lysolecithins and α-glycerylphosphoryl-holine. This means the enzyme in general splits choline from lecithins and derivatives f lecithins which contain choline.

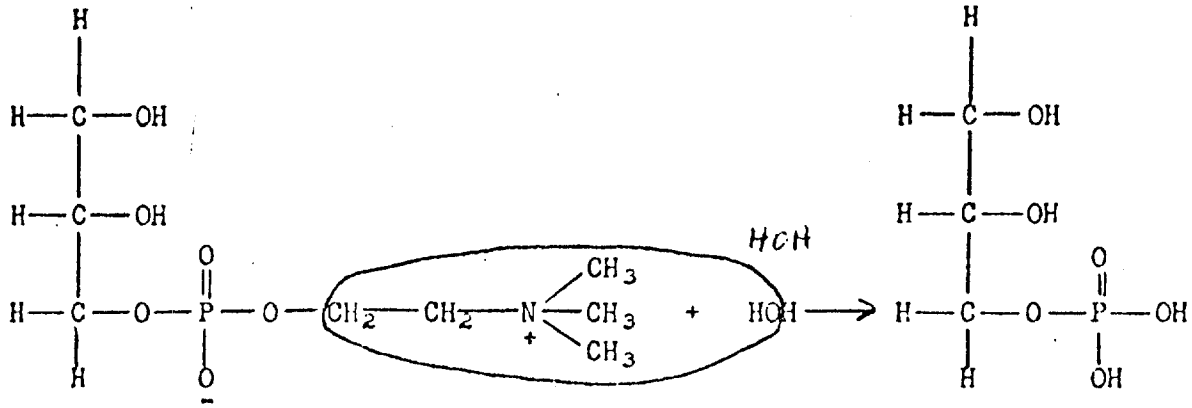


Lecithin

Phosphatidic acid

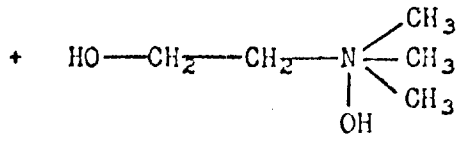


Choline



α-Glycerylphosphorylcholine

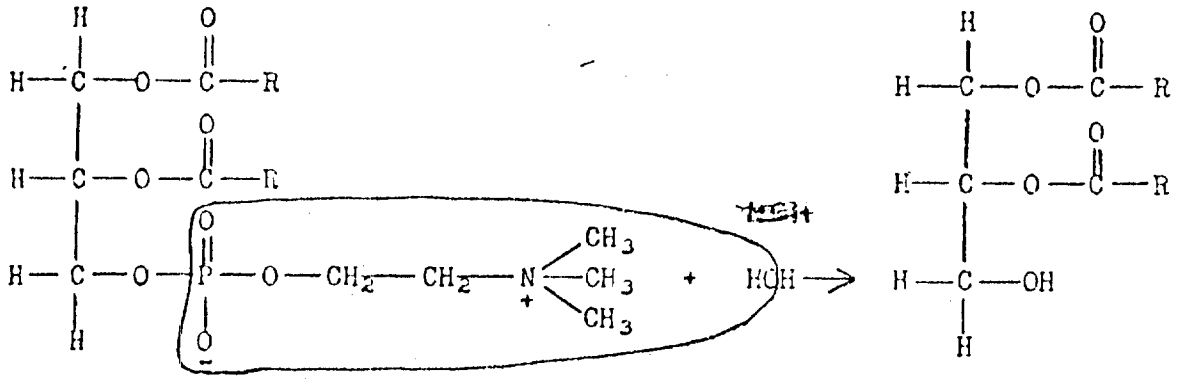
α-Glyceryl-phosphoric acid



Choline

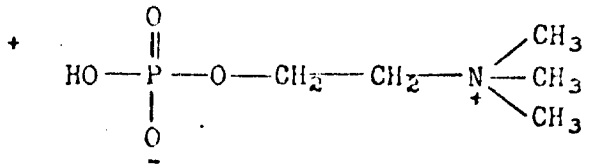
Lecithinase C occurs in intestinal juice, pancreas, and other organs.

IV. Lecithinase D. This enzyme splits choline phosphoric acid from lecithin and presumably lecithin derivatives containing the choline phosphoric acid group (lysolecithin and α-glycerylphosphorylcholine).



Lecithin

A diglyceride



Choline phosphoric acid (phosphorylcholine)

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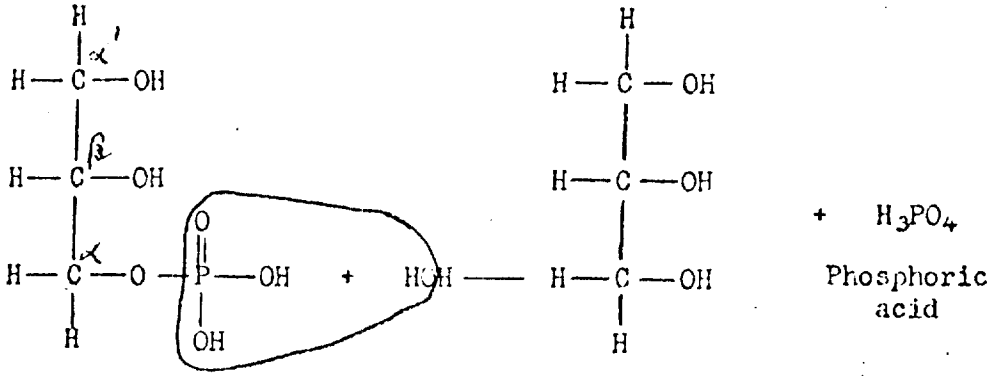
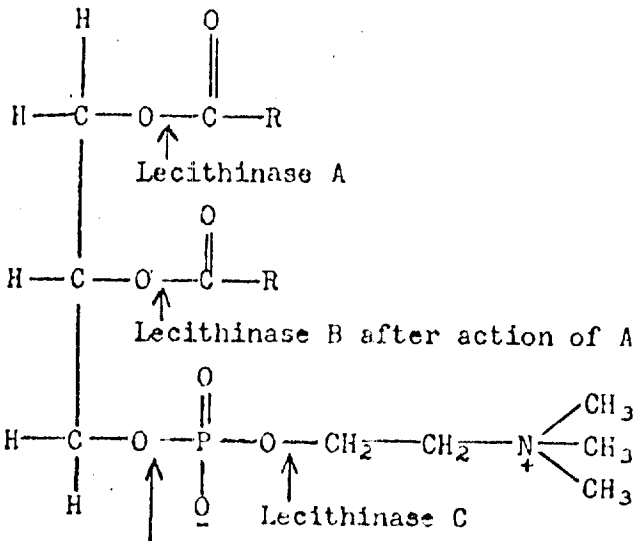
V. Phosphatase. This enzyme splits phosphoric acid from glycerophosphoric acid:



Phosphatases are widespread in digestive juice, tissues, and body fluids.

The above discussion indicates the complexity of the digestive processes by which the lecithins and related substances are broken down in the intestine and ssues into their components. The phosphoglycerides containing ethanolamine and serine are apparently broken down by the same enzymes.

By way of summary of the digestive process, the lecithin molecule is shown below with the various points of hydrolysis by different enzymes:



The various products obtained in the digestion of soy bean phosphatides according to the above processes should be:

I. From the phosphatidyl choline fraction (true lecithins):

Lysolecithin, glycerylphosphorylcholine, phosphatidic acid, choline-phosphoric acid, fatty acids, choline, α-glycerol phosphoric acid, glycerol and phosphoric acid. If the digestion were to go to completion the products would only be glycerol, fatty acids, choline, and phosphoric acid.

II. From the phosphatidyl ethanolamine fraction:

Lysophosphatidyl ethanolamine, glycerylphosphoryl ethanolamine, phosphatidic acid, ethanolamine phosphoric acid, fatty acids, ethanolamine, α-glycerol phosphoric acid, glycerol, and phosphoric acid. Complete digestion would give only glycerol, fatty acids, ethanolamine, and phosphoric acid.

The small amount of phosphatidyl serine presumably would give products analogous to those from phosphatidyl ethanolamine.

III. From the phosphoinositides (inositol-containing phosphatides):

Essentially nothing appears to be known about the specific enzymatic digestion of this class of substances. Assuming that intestinal digestion is effective we would expect to have as digestion products the following:

Inositol, phosphoric acid, galactose, glycerol, ethanolamine, palmitic, stearic, arachidic, hexadecenoic, oleic, linoleic, linolenic, and a small amount of other fatty acids, and possibly an unidentified primary amine.

Thus it is seen that the partial and complete digestion of the phosphatides of soy beans would yield a large group of substances, some of which have important biological functions. Among the latter may be mentioned:

Choline, inositol, phosphoric acid, unsaturated fatty acids (particularly linoleic and linolenic acids) and ethanolamine.

Absorption of phosphatides and their digestion products from the intestine.

Not much work has been directed toward the absorption of phosphatides. Artom

nd Swanson (J. Biol. Chem., 175, 871, 1948) fed emulsions of rat liver phosphatides
eled with radioactive P^{32} to rats, and also as controls they gave emulsions of
onlabeled phosphatides mixed with inorganic phosphate or glycerophosphate labeled
ith P^{32}. After 3-6 hours the radioactivity and phosphorus content of plasma and
iver fractions were determined. From the results it was concluded that the
dministered phospholipids were absorbed at various stages of hydrolysis, but that
detectable portion can be absorbed as the intact molecule. These phosphatides
ere chiefly phosphoglycerides, and the experiment throws no light upon the absorption
f the inositol-containing phosphatides. Also, the experiment did not differentiate
etween the absorption of unchanged phosphatide and phosphatide from which one fatty
cid or the nitrogen base had been split.

Bloom, Kiyasu, Reinhardt, and Chaikoff (Am. J. Physiol., 177, 84, 1954) fed
losynthesized rat liver phosphatides containing C^{14} labeled palmitic acid and
vered 15-30 per cent of the labeled fatty acids in a phosphatide fraction of
ntestinal lymph. Under the same conditions only 2-7 per cent of C^{14} labeled
almitic acid in tripalmitin (triglyceride) was recovered in the lymph phosphatides.
his result shows that as much as 20-25 per cent of phosphatide (phosphatidyl choline
true lecithin) and phosphatidyl ethanolamine) may be absorbed without breakdown of
he molecule.

The absorption of phosphatides from the intestine into the lymph is of interest
connection with phosphatide synthesis by intestinal cells. It is certain that
rtially hydrolyzed products of phosphatides pass into the intestinal wall and are
ere reconverted to phosphatides, thus bringing about interchange of phosphatide
mponents.

Reiser and associates (J. Biol. Chem., 194, 131, 1952) fed triglycerides
beled with double bonds in the fatty acid (linoleic) and C^{14} in the glycerol
idue to rats and determined the distributions of C^{14} glycerol and linoleic acid
the lymph glycerides (fats) and phosphatides. From the results they concluded

that:

1. Between 25 and 45 per cent of the ingested triglyceride was completely hydrolyzed to fatty acids.

2. The glycerol formed was not utilized by the intestinal cells to form lymph glycerides but followed an independent pathway of metabolism.

3. 55 to 75 per cent of the ingested triglycerides was hydrolyzed to monoglycerides and fatty acids.

4. Half of the lymph phosphatide formed from the ingested fat by the intestinal cells was formed from fatty acids hydrolyzed from the triglyceride and glycerol of the intestinal cells, while half was formed from absorbed monoglycerides by attaching fatty acid, choline and phosphoric acid.

Borgström (Acta Chem. Scand., 5, 643, 1951; Acta Physiol. Scand., 25, 140, 1952) gave C^{14} labeled palmitic and stearic acids in corn oil to rats and found out 90 per cent of the labeled acids in the triglycerides and as much as 10 per cent in the phosphatides of lymph. Thus during fat absorption the intestinal wall supplies an important part of the plasma phosphatides through synthesis.

Formerly it was thought that a considerable proportion of fat absorption in the intestine involved phosphatide formation from the glycerol and fatty acids during passage through the intestinal wall, but results as obtained above indicate that quantitatively this is of less importance than previously supposed. However, it has been reported (Frazer, Bull. Soc. Chim. biol., 33, 961, 1951) that administration of choline may increase fat absorption as much as 50 per cent. Tidwell and Tidwell and Nagler (J. Biol. Chem., 182, 405, 1950; Federation Proc., 10, 258, 1951; 11, 298, 1952) also found choline given with fat to increase fat absorption. Of particular interest to us is the report by Adlersberg and Sobotka (J. Nutrition, 25 255, 1943) that the administration of lecithin increases the rate of fat absorption humans. This also has been found to be the case in animals (Deuel and associates, J. Nutrition, 33, 177, 1947). Adlersberg and Sobotka gave 1 g. of butter fat per

kg. of body weight to normal persons with and without 10-15 g. of commercial soy bean lecithin. These substances were given 12 hrs. after the last meal (postabsorptive fasting state). The total serum lipids were then determined 4-5 hours after receiving the fat or fat plus lecithin. It was found (average of 5 cases) that the lecithin caused serum lipids to rise an average of 71 per cent above the fasting level, whereas without the lecithin the rise was only 32 per cent. In 3 cases of sprue which showed essentially no serum lipid increase after fat ingestion, the addition of lecithin caused serum lipids to rise 27 per cent above the fasting level. These workers also gave 180,000 units of vitamin A to 7 normal subjects with and without lecithin and 4 hours later determined serum vitamin A levels. Without lecithin serum vitamin A rose 41 per cent above fasting, while with it serum vitamin A rose 212 per cent above the fasting level! A similar increase was noted in cases of sprue.

This work shows a marked effect of soy phosphatides upon absorption in both the normal human and in sprue cases where intestinal absorption is greatly decreased. It appears that soy bean lecithin should be of definite value in the numerous gastrointestinal diseases and in geriatrics where absorption is deficient. This would appear to be very concrete evidence for therapeutic value of the Glidden product.

It appears probable that this effect of lecithin upon absorption is due to the emulsifying action of the lecithin upon the fats which provides better contact with the fat digesting enzymes and also renders fats and their hydrolysis products more permeable to the intestinal membranes.

Synthesis of phosphatides in the body

The universal distribution of phosphatides in living tissues attests to their fundamental biochemical importance. That each tissue has the capacity to synthesize its own phosphatides appears well established from the many experiments carried out with isotopes (see the review by Artom, Phosphorus Metabolism, Vol. II, page 203. The Johns Hopkins Press, Baltimore, 1952; also the review by Zilversmit, Annual Review of Biochemistry, 24, 157, 1955).

Blood phosphatides are derived largely from the liver (Chaikoff and associates, J. Biol. Chem., 150, 47, 1943), and to a smaller extent from intestinal absorption and synthesis, and the liver represents the primary site of removal of phosphatides from plasma.

The rates at which phosphatides are synthesized and broken down (turnover rates) in tissues are relatively fast generally, though brain phosphatides appear to change slowly. The relative order of turnover rates for rat tissues appear to be: liver > intestine > kidney > muscle > brain.

It has been shown that various substances and factors influence the rate of phosphatide turnover. Choline feeding has been found to increase the rate of lecithin turnover in the livers of dogs (Chaikoff and associates, J. Biol. Chem., 176, 193, 1948), and this appears to be due to metabolic activity within the liver cells. The level of liver lecithin was increased. The amino acids methionine, cystine, and cysteine also increase the turnover rate, as does betaine (J. Biol. Chem., 135, 359, 1940; 130, 593, 1939).

Cholesterol feeding decreases the turnover rate of liver phosphatides (J. Biol. Chem., 128, 735, 1939) and causes fatty livers. This effect is counteracted by feeding choline.

Artom and Cornatzer (J. Biol. Chem., 165, 395, 1946) showed that a large dose of choline administered with fat to rats caused increased formation of phosphatides in the intestinal cells, suggesting that the supply of choline to these cells may be a limiting factor in this synthesis.

The liver and kidneys of choline deficient rats are deficient in phosphatides (Patterson and McHenry, J. Biol. Chem., 145, 207, 1942).

Artom and Marziani (Bull. soc. chim. biol., 6, 713, 1924) found the removal of ovaries from rabbits causes a decrease in liver phosphatides, and thyroidectomy and pancreatectomy produce similar results. Schmidt (Am. J. Physiol., 111, 138, 1935) found that the injection of thyroxin causes a decrease in liver phosphatides but an

increase in fatty acids from other lipids. In skeletal muscle phosphatides increased and fatty acids from other lipids decreased. In blood both increased.

Tumors have been found (Chaikoff, Physiol. Revs., 22, 305, 1942) to incorporate P^{32} into phosphatides more rapidly than other tissues.

The influence of various hormones, diet, and other substances and factors upon phosphatide formation in different tissues of different animals constitutes a very complex and often confusing picture, and to go into it further here does not seem profitable. Reviews of this phase of phosphatides are given by Artom (Phosphorus Metabolism, Vol. II, page 203) and by Zilversmit (Annual Review of Biochemistry, 24, 157, 1955).

The actual chemical reactions utilized by tissues in the synthesis of phosphatides (Phosphoglycerides) have been partially worked out.

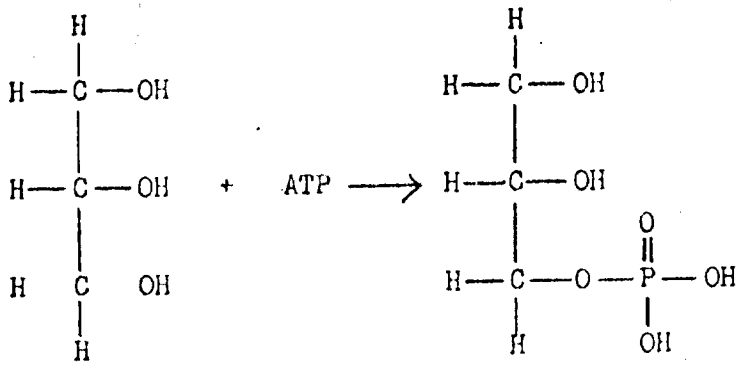
The presence in tissues of many of the molecules of which phosphatides are composed is well established. These substances undoubtedly represent both products being formed and utilized by tissues in the synthesis of phosphatides, and substances formed in the breakdown of phosphatides. Such substances found in tissues are:

Glycerol, fatty acids, choline, inorganic phosphate, glycerol phosphate, phosphoryl choline (choline phosphate), phosphoryl ethanolamine (ethanolamine phosphate), glyceryl phosphoryl choline, glyceryl phosphoryl ethanolamine, lignoceryl sphingosine, sphingosine choline phosphate, serine, and inositol.

Isotopically labeled inorganic phosphate, glycerol, fatty acids, choline, ethanolamine, and serine have all been shown to be incorporated into the phosphatide fractions of tissues.

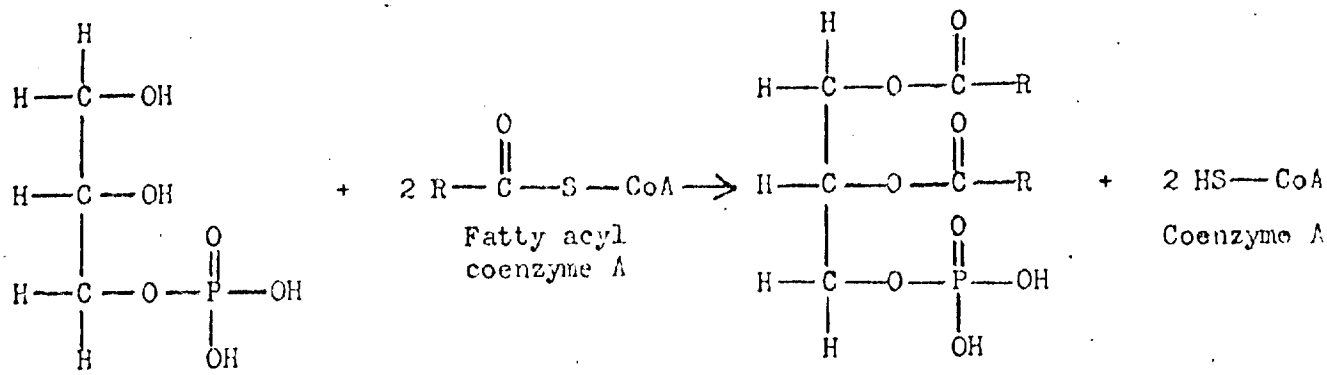
Kornberg and Pricer (J. Biol. Chem., 204, 345, 1953) and Kennedy and associates (J. Am. Chem. Soc., 75, 249, 1953; J. Biol. Chem., 201, 399, 1953; 209, 525, 1954; Federation Proc., 13, 241, 1954) have provided evidence for specific chemical processes by which tissues form phosphatides of the phosphoglyceride class (phosphatidyl cholines, phosphatidyl ethanolamines, and phosphatidyl serines). These workers used rat liver

enzyme preparations and liver mitochondria. According to this evidence synthesis proceeds as follows:



Glycerol

α-Glycerol phosphate

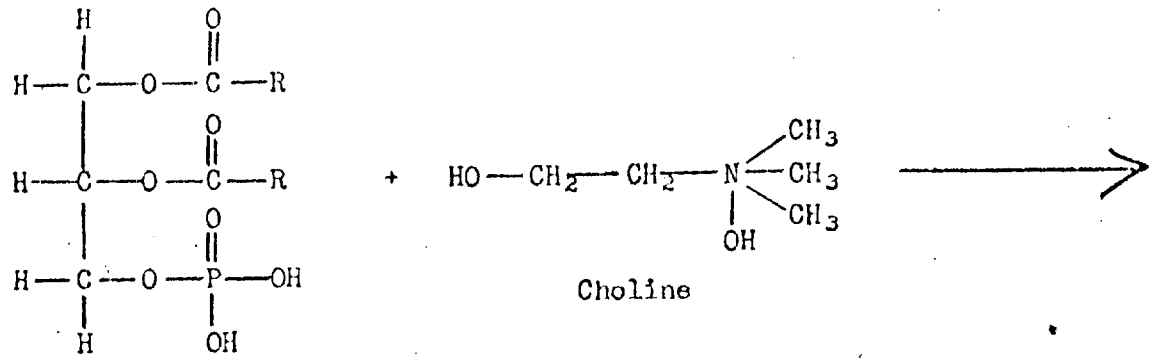


α-Glycerol phosphate

Fatty acyl coenzyme A

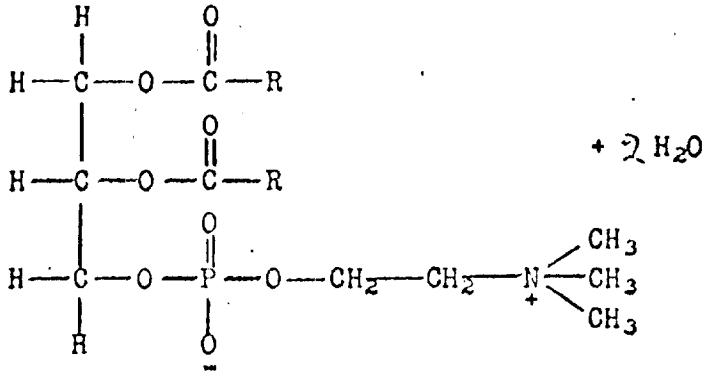
Phosphatidic acid

Coenzyme A



Phosphatidic acid

Choline



Phosphatidyl choline

Substitution of ethanolamine or serine for choline should give the other two classes of phosphoglycerides.

While other pathways of synthesis possibly may be utilized, the above process appears to be established. Nothing is known of the mechanisms involved in the synthesis of the complex inositol phosphatides.

It is obvious that phosphatides may be broken down in tissues into their component molecules and these reassembled into other phosphatides, giving a very complex situation.

Tissue phosphatides

The quantities of phosphatides present in blood and other tissues vary with physiological states, but for each type of tissue there is a rather characteristic range of values. The methods of analysis used by different workers have varied and have given variation in values characteristic of the methods. This fact combined with actual changes in tissue phosphatides with physiological and individual variations lead to a rather wide spread in values.

I. Human blood

The table below summarizes values obtained by chief workers in the field, each entry representing the values obtained by a worker or group.

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Serum, plasma or corpuscles	Total phosphatides mg. %	Lecithins* % of total	Cephalins** % of total	Sphingomyelins % of total	Dietary state of subjects
Serum (6)	226	47	42	11	Postabsorptiv
Serum (12)	185		22		Postabsorptiv
Serum (2)	229	60	27	13	Not stated
Plasma (4)	189	52	29	19	Not stated
Plasma (unknown no.)	204	61	22	17	Not stated
Plasma (16)	152		20		Postabsorptiv
Plasma (20)	145	13	47	40	Fasting
Plasma (11)	235		3***		Postabsorptiv
Plasma (unknown no.)	153	55	28	12	Not stated
Serum (2)	184		59		Not stated
Erythrocytes (4)	317	24	60	50	Not stated
hrocytes (20)	196	16	60	24	Fasting
Erythrocyte stroma (4)	10	20	50	30	Not stated
Leucocytes (22)	844±241				Not stated

*Phosphatidyl cholines.

**Phosphatidyl ethanolamines chiefly, with some phosphatidyl serines, and inositol phosphatides if present.

***The low values for cephalins, 3 per cent of total, obtained by Chaikoff and associates is noteworthy. These workers (J. Biol. Chem., 156, 385, 1944) determined the lecithin and cephalin distribution on the basis of choline to phosphorus ratio, and found 97 per cent of the plasma phosphatides to contain choline (lecithins and sphingomyelins), showing the absence of appreciable amounts of the cephalin fraction. Later work by Sinclair (J. Biol. Chem., 174, 343, 355, 1948) corroborated this work of Chaikoff and associates, and it appears that much of their work on the distribution of ~~different~~ phosphatide fractions in blood is questionable. This shows again the unsatisfactory state of our knowledge relative to the phosphatides, and the necessity of improving analytical methods.

It has been found that a large proportion of the phosphatides of plasma is associated with plasma proteins as lipoprotein complexes. These lipoproteins appear to be formed chiefly with the α_1- and β_1-globulin fractions of plasma proteins. Besides containing much phosphatide, these lipoproteins may contain up to 20 per cent cholesterol, and some fatty acids. Edsall (Advances in Protein Chemistry III, Academic Press, New York, 1947) states that the β_1 lipoprotein contains 75 per cent lipid, but that it dissolves up to 10 per cent in dilute aqueous salt solutions. The molecular weight of this complex appears to be about 1,000,000. Most of the lipid of lipoproteins may be extracted with fat solvents, indicating loose bonding. These lipoprotein complexes are important in keeping the lipids dissolved in plasma. Undoubtedly similar complexes between phosphatides (and other lipids) and proteins exist generally in tissues.

Kaucher and associates (Arch. Biochem., 3, 203, 1943) have studied the phosphatide content of various beef organs, and reported their results on the basis of per cent of dry weight of the organ.

Organ	Total phosphatide	Lecithins	Cephalins	Sphingomyelins
Brain	26.37	7.05	14.35	4.96
Liver	16.22	8.86	6.59	0.76
Kidney	10.32	5.62	3.02	1.66
Heart	9.83	3.96	5.34	0.52
Lung	9.78	3.60	3.90	2.27
Thymus	6.71	3.28	2.72	0.70
Muscle	3.24	1.84	1.19	0.20
Egg, chicken	13.73	9.95	3.44	0.34

Insert a of interest

Bloor made an extensive study of the phosphatide to cholesterol ratio in muscles of many animals and found this ratio to be highest for muscles with the highest activity, and also, that exercise of a given muscle increases the ratio (Bloor, Biochemistry of the Fatty Acids, Reinhold Publishing Corporation, 1943, pp. 200-210).

The above values for phosphatides in tissues, the fact that the most active muscles (such as heart) contain the most phosphatide, and the fact that the tissues

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of starved animals still contain much phosphatide (fats practically gone) point up the importance of phosphatides as fundamental constituents of tissue machinery.

Some pathological relations of the phosphatides

While undoubtedly a large proportion of pathological states are associated with changes in phosphatide distribution, yet at the present time our information in this field is severely limited. In the following discussion an attempt is made to briefly outline some of the relations of phosphatides to abnormal states. These states vary from what may be considered exaggerated physiological to patent pathological conditions. A partial review is to be found in "The Phosphatides", by Wittcoff, pp. 424-442, Reinhold Publishing Corporation, New York, 1951.

A. Fatty Livers

While the normal mammalian liver contains about 5 per cent lipids, in a number of pathological and physiological disturbances the lipid content may rise to 25 to 30 per cent or higher, and under these conditions "fatty livers" are said to exist.

Fatty livers in general contain more cholesterol (chiefly as esters with fatty acids), more neutral fat, and less phosphatides than normal, though the proportions of lipids present vary widely under different conditions.

1. Liver injury

Cirrhosis of the liver associated with syphilis, alcoholism, and prolonged malarial fevers may show marked fat infiltration and deposition in the liver. Fat deposition is particularly copious in acute yellow atrophy, where it may amount to 50 per cent of the weight of the liver.

Injury to the liver by poisons such as carbon tetrachloride and phosphorus may lead to fatty infiltration of the liver.

In all of the above cases the fatty livers appear to result because injury of the liver decreases its capacity to metabolize the lipids brought to it.

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2. Diabetes

In diabetes, because of the defect in carbohydrate metabolism, much of the energy must be derived from fats, and this is true of the liver as well as most other tissues (brain excepted apparently). In this condition depot lipids (mainly fat) are mobilized into the blood stream and these with dietary lipids are brought to the liver in much exaggerated quantities, quantities which exceed the capacity of the liver to metabolize them, with the result that the liver lipid content increases to produce fatty livers. After the diabetic state is relieved by insulin, the excess lipid disappears from the liver. This condition represents fatty livers due to an exaggerated physiological condition.

3. Carbohydrate deprivation

Fatty livers have been caused in mice and rats by starvation, in which blood lipids markedly increase, or by feeding diets high in fat and low in carbohydrate. Carnivorous animals, such as the dog, do not show starvation fatty livers. In these cases of fatty livers the increased lipid in the liver is due to the increased load of lipid imposed upon the liver for metabolism, an exaggerated physiological condition.

The conditions causing fatty livers outlined above do not appear to involve deranged phosphatide metabolism. Most of the nonhormonal causes of fatty livers given below appear to directly or indirectly concern phosphatides.

4. Unsaturated fatty acid deficiency

Animals fed diets deficient in the so-called essential fatty acids (linoleic, linolenic, arachidonic, abundantly supplied by commercial soy bean lecithin) develop fatty livers. Since liver phosphatides contain a rather large proportion of such unsaturated acids (which the body cannot make), a dietary deficiency of these acids inhibits liver phosphatide formation. The metabolism of fat in the liver is in some way associated with liver phosphatides, and any condition which decreases the synthesis and utilization of phosphatides in the liver decreases fat oxidation and

metabolism in the liver (probably in other tissues also, E.S.W.), and may lead to the accumulation of excess lipids in the liver, especially fat.

5. Choline deficiency

It has long been known that diets deficient in choline lead to fatty livers, which are relieved by giving choline or lecithin (which provides choline). Also, the provision of adequate methionine prevents fatty livers due to choline deficiency, and this is due to the fact that if the body has plenty of methionine it can synthesize sufficient choline to prevent fatty liver. The fatty livers due to choline deficiency undoubtedly are caused by inability to synthesize needed phosphatides (phosphatidyl cholines and sphingomyelins). Perlman and Chaikoff (J. Biol. Chem., 127, 211, 1939) showed that both choline and methionine increase the rate of phosphatide turnover in the livers of dogs receiving P^{32} inorganic phosphate.

6. Amino acid deficiency

Sydenstricker and associates (J. Biol. Chem., 200, 867, 875, 883, 1953) observed fatty livers in rats on diets deficient in lysine or threonine, which are controlled by adequate amounts of these amino acids. It has been found that threonine deficiency depresses the rate of phosphatide and nucleic acid synthesis in the liver, and administration of threonine restores the synthesis. Here again the role of phosphatides in relation to fat metabolism in the liver is evident.

7. Excess cystine feeding

Fatty livers may be produced by feeding excessive amounts of cystine in the absence of adequate choline or methionine. This appears to be due to the excess cystine causing increased tissue protein synthesis and using up the supply of available methionine for this purpose to an extent which leaves an inadequate supply for the formation of choline and phosphatide synthesis.

8. Cholesterol feeding

Very fatty livers have been produced in rats by feeding cholesterol. The livers contain much cholesterol, and an excess of neutral fat. Choline administration

tends to alleviate the fat infiltration but is much less effective upon the cholesterol. Perlman and Chaikoff (J. Biol. Chem., 127, 211, 1939) showed that cholesterol feeding (high liver cholesterol) decreases the rate of phosphatide turnover in the liver, which is corrected by choline.

It has been found that a combination of choline and inositol is more effective than choline alone in controlling fatty livers due to cholesterol feeding. Here apparently the fatty livers are due to insufficient phosphatide synthesis and turnover in the liver.

9. Excess biotin

It has been shown that fatty livers may be caused by feeding an excessive amount of the vitamin biotin. This condition is not relieved by choline but is by inositol. The theory relative to such fatty livers is that excess biotin somehow increases the demand for inositol and depletes the quantity available for the formation of inositol phosphatides. This is only a theory.

10. Pyridoxine deficiency

Engel (J. Nutrition, 24, 175, 1942) has described conditions under which pyridoxine deficiency leads to fatty livers which are resistant to choline but relieved by inositol. The deficiency may cause an increased demand for inositol thereby depleting the supply for inositol phosphatide synthesis. This is an unsubstantiated theory only.

11. Guanidoacetic acid feeding

Stetten and Grail (J. Biol. Chem., 144, 175, 1942) produced extremely fatty livers by feeding guanidoacetic acid to rats, even when receiving choline. Apparently the choline was decomposed to provide methyl groups to methylate the choline to creatine, leaving an inadequate supply for phosphatide synthesis.

Other conditions may lead to fatty livers (see West and Todd, Textbook of Biochemistry, 2nd ed., pp. 898-900) but those given above appear sufficient to stress the extreme importance of phosphatides in liver lipid metabolism.

B. Lipidoses

There are a number of pathological conditions in which abnormal amounts of certain lipids accumulate in organs or subcutaneously. Apparently in only one of these are phosphatides primarily involved, and this is Niemann-Pick's disease in which lipides accumulate in any of the organs, but especially in the spleen, liver, lymph nodes, bone marrow, and central nervous system. These lipid accumulations contain much sphingomyelins, though the glycerophosphatides may also be markedly increased. The causes for Niemann-Pick's disease are unknown.

The xanthomatoses are characterized by the accumulation of lipids subcutaneously in large spongy masses. The lipid deposits were found by Eckstein and Wile (J. Biol. Chem., 87, 311, 1930) to contain 48.8 per cent cholesterol and 8.1 per cent phosphatide. In so-called primary xanthomatoses total blood lipids are high, with high values for both cholesterol and phosphatides. Adlersberg and Sobotka (J. Mt. Sinai Hosp., N. Y., 9, 955, 1943) reported on five cases of xanthomatosis in which the hypercholesterolemia was alleviated by prolonged feeding of phosphatides. Interruption of this therapy caused the lipemia to return.

C. Other conditions

Diseases of the liver

Elevation in blood phosphatides have been found in hepatitis, however cases have been observed in which the phosphatide level was low or normal.

Diseases of the kidneys

Diseases of the kidneys like those of the liver are usually associated with increases in blood lipids (lipemia). In the nephrotic syndrome with increased total blood lipides, the phosphatide content appears to increase in proportion.

Diseases of the pancreas

In diseases of the pancreas which prevent the production of digestive enzymes, one of the most common characteristics is failure to digest and absorb fats leading to much lipids in the feces -- steatorrhea. The blood lipid level falls with reduction

in fats, cholesterol, and phosphatides due largely to failure of absorption, and probably also failure of synthesis due to decreased supplies of substances necessary for synthesis. The improvement in fat absorption due to the administration of soy bean lecithin in cases of sprue with steatorrhea as observed by Adlersberg and Sobotka has already been noted earlier in this discussion.

Diseases of the blood

In diseases of the blood leading to various types of anemia Bloor and MacPherson (J. Biol. Chem., 31, 79, 1917) showed a decrease in blood cholesterol and phosphatides, the change being much more marked in the plasma than in the cells. Williams and coworkers (J. Biol. Chem., 118, 599, 1937; J. Lab. Clin. Med., 26, 996, 1941) found in pernicious anemia that the cholesterol esters increase considerably and the phosphatides decrease slightly in the erythrocytes, while all of the phosphatide fractions in the plasma are far below normal. After treatment of pernicious anemia Kirk (Am. J. Med. Sci., 196, 648, 1938) observed a marked increase in the ether insoluble phosphatides of plasma.

It has been observed that the phosphatides of spinal fluid are increased during pernicious anemia (Semana Méd. (Buenos Aires) I, 403, 1948; Chemical Abstracts, 42, 6923, 1948).

Diseases of the blood vessels

Because lipids, particularly cholesterol, accumulate in the atheromatous patches of blood vessels in arteriosclerosis, considerable attention has been directed to it. Atherosclerosis has been produced in birds (Arch. Path., 38, 46, 1944) and in rabbits (Arch. Path., 18, 473, 600, 827, 1934) by feeding cholesterol. This is associated with an increase in both cholesterol and phosphatides of blood (Biol. Zhur., 4, 507, 1935; Chemical Abstracts, 32, 7561, 1938). Chaikoff and associates (J. Biol. Chem., 179, 113, 1949) have shown that arterial tissue is capable of synthesizing fatty acids from C^{14} acetate, and also is capable of incorporating labeled (P^{32}) inorganic phosphate into phosphatides. This indicates that synthesis

of lipids by blood vessels may contribute to atheromatous lesions.

Downs (Am. Med., 41, 460, 1935) and Kesten and Silbowitz (Proc. Soc. Exp. Biol. Med., 49, 71, 1942) reported that the ingestion of phosphatides diminishes both the hypercholesterolemia and the arteriosclerosis resulting from feeding cholesterol to rabbits.

Hypertension is often associated with arteriosclerosis, and increases in all lipid fractions, including phosphatides, have been reported in hypertension (Klin. Wochschr., 11, 886, 1932). However, Peters and Man (J. Clin. Invest., 22, 715, 721, 1943) are of the opinion that blood lipids in hypertension are not disturbed unless there are associated causes such as nephritis.

Geyer and associates (J. Lab. Clin. Med., 34, 688, 1949) isolated from commercial soy bean phosphatides a fraction which showed appreciable blood pressure lowering action in man. It appeared necessary to allow the phosphatides to stand in air for development of this property.

Diseases of the central nervous system

Such diseases appear to have little effect upon blood phosphatides or other lipids though Roeder (Z. ges. Neurol. Psychiat., 168, 519, 1940) reported the phosphatide content of spinal fluid to be decreased in cases of schizophrenia.

Tumors and neoplasms

Actively growing tumors more rapidly synthesize and use phosphatides (higher turnover rates) than do normal tissues, as shown by increased rates of incorporation of P^{32} inorganic phosphate (J. Biol. Chem., 128, 631, 1939; 133, 319, 1940; Am. J. Cancer, 40, 235, 1940). Different tumors show widely different rates of phosphatide turnover.

Tuberculosis

The phosphatides containing phthioic acid from the tubercle bacillus were shown by Sabin and associates (J. Exp. Med., 52, suppl. No. 3, 3, 1930; Physiol. Rev., 12, 141, 1932) to cause the formation of typical tubercles (defense reaction).

Increases, no change, and decreases in blood phosphatides of tuberculosis patients have been reported. The situation is uncertain.

Tompkins reported (Amer. Rev. Tuberc., 33, 625, 1936) that egg yolk lecithin, injected before or after intravenous inoculation of rabbits with bovine tubercle bacillus, caused more rapid and extensive healing of pulmonary lesions than in the control animals. This may have been due to phosphatide activation of enzymes which break down the tubercle bacillus.

Diseases of the heart

Ide (Hukuoka Acta Med., 33, 35, 1940; Chemical Abstracts, 36, 820, 1942) reported the phosphatide and cholesterol of heart muscle to decrease in functional cardiac disturbances. The plasma lipids, including the phosphatides, are generally increased in coronary sclerosis.

Diseases of the skin

Hansen (Proc. Soc. Exp. Biol. Med., 41, 205, 1939) reported the blood phosphatide fatty acids of eczematous infants to be more saturated than normal. Phosphatides have been used as therapeutic agents for psoriasis (J. Investigative Dermatol., 5, 321, 1942).

Avitaminoses

Soy bean lecithin (phosphatides) have been shown to increase vitamin A absorption in man (Adlersberg and Sobotka, J. Nutrition, 25, 255, 1943), and Slanetz and Scharf (J. Nutrition, 30, 239, 1945) obtained evidence that a factor in commercial soy bean phosphatide influences the blood levels, storage and utilization of vitamin A in the rat. Esh and associates (J. Dairy Sci., 31, 461, 1948) showed that soy bean phosphatides increase the absorption of vitamin A in the cow and increases transmission of colostral vitamin A to the calf.

György (N. Y. Acad. Sci., 49, 525, 1948) states that pantothenic acid deficiency causes hemorrhagic syndromes which may be related to a general blood dyscrasia caused by disturbance in the utilization and synthesis of phosphatides.

Palladin and Kudrajawzowa (Biochem. Z., 154, 104, 1924) reported that rabbits deficient in vitamin B show decreased blood neutral fat and free cholesterol, and increased amounts of phosphatides, cholesterol esters and fatty acids.

Hongo (Sei-i-kwai Med. J., 53, No. 5, 1, 1934; Chemical Abstracts, 29, 3381, 1935) reported that the tissues of guinea pigs on vitamin C-deficient diets contain generally more lecithin and free cholesterol than tissues of normal animals. Sueyoshi and Mitimoto (J. Biochem. (Japan), 30, 155, 1939) found the tissues of scorbutic guinea pigs to contain less than normal amounts of highly unsaturated cephalins. Since cephalin phosphatides promote blood coagulation, it may be that decrease in this fraction is causatively related to the capillary bleeding observed in scurvy.

While no variations from normal have been found in vitamin D-deficient animals (rats), Branson and associates (Science, 106, 637, 1947), however, reported that vitamin D facilitates the incorporation of inorganic phosphate into phosphatides.

In connection with this report it is very interesting that the phosphorus of soy bean phosphatides is utilized as efficiently as inorganic phosphate for bone calcification (Krieger and associates, J. Nutrition, 21, 213, 1941). This indicates that the phosphate component of soy phosphatides is efficiently split off and utilized.

Diseases of the thyroid gland

Hyperthyroidism is associated with decreased serum lipids, including phosphatides, while in hypothyroidism serum lipids, including phosphatides, are increased.

Pneumonia

It appears that febrile diseases generally cause a decrease in serum lipids. Pneumonia is characterized by decreased blood phosphatides and fatty acids, with least effect upon the fatty acids (Stoesser and McQuarrie, Proc. Soc. Exp. Biol. Med., 46, 83, 1941; Am. J. Diseases Children, 49, 658, 1935).

Harris and associates (Am. J. Syphilis Neurol., 18, 333, 1934) reported that

the resolution of syphilitic lesions, produced experimentally in rabbits, is hastened by lecithin injection. This was attributed to stimulation by lecithin of the phagocytic mononuclear cells.

Progressive paralysis

Carbone and Pighini (Biochem. Z., 46, 450, 1912) and Singer (Biochem. Z., 198, 340, 1928) reported a decrease in brain phosphatides in cases of progressive paralysis.

Multiple sclerosis

Weil (J. Neuropathol. Exp. Neurol., 7, 453, 1948) found the phosphatide content of the white and gray matter of brain to be markedly decreased in patients with multiple sclerosis. Miner reported improvement in 80 per cent of 130 cases of multiple sclerosis treated with lecithin (Münch. Med. Wochrschr., 86, 1038, 1939).

The above discussion of phosphatides and pathological states shows a multitude of relations, and indicates the great importance of phosphatides to life processes. Only the surface of the problem has been touched, and we shall have to wait for some time before the reasons underlying the relations of phosphatides to pathology are understood. First, we must have a much clearer picture of the normal functions of phosphatides in the body.

Biological functions and actions of phosphatides

The following discussion will attempt to outline present concepts as to the functions and biological actions of phosphatides. Some of these appear to be well established, while the evidence for others is suggestive but not yet conclusive.

I. Phosphatides are universally found as components of living cells. They are constituents of metabolically important structures such as the mitochondria.

II. Phosphatides, through absorption in the intestine and synthesis in the intestinal cells, serve in bringing fatty acids and the other components of phosphatides (glycerol, phosphate, choline, ethanolamine, etc.) into the blood stream, and tissues where they are utilized. The actual proportion of food fatty acids which enters

intestinal cells and is there synthesized into phosphatides for entrance into the lymph and blood stream is relatively small. Thus, the phosphatides have been found to play a much less important role in fatty acid absorption than was formerly thought.

III. Dietary phosphatides, such as soy bean lecithin, have been shown to markedly increase the rate of absorption of fats and vitamin A by both normal humans and patients in which steatorrhea (failure of fat digestion and absorption) exists. This effect is probably largely related to the emulsifying action of the phosphatides in promoting better contact between enzymes and fats, and also in permitting better direct absorption of the highly emulsified fat and vitamin A. The phosphatides, obviously, should be taken with the food.

IV. The phosphatides play an important role as constituents of the lipoprotein complexes of plasma in which much of the blood lipids is loosely held and transported. These lipoproteins are soluble in the plasma, whereas without such complexes the lipids could not be maintained in solution.

V. The phosphatides are components of the complex thromboplastic lipoproteins concerned with blood coagulation. Chargaff and associates (J. Biol. Chem., 156, 161, 1944; 161, 389, 1945) isolated the thromboplastic proteins from beef lung, and human lung and placenta, and while all of these preparations appeared to be similar, their identity was not established. These workers examined the lipid fraction of the thromboplastic lipoprotein isolated from beef lung by differential centrifugation, and which was homogeneous in the ultracentrifuge. They found 40 to 45 per cent lipids in it distributed as follows (per cent of total lipids): cholesterol, 19; neutral fat, 18; lecithin fraction, 26; cephalin fraction, 25; sphingomyelin fraction, 12; and acetal phosphatides, 1.5. Chargaff (J. Biol. Chem., 155, 387, 1944) was unable to demonstrate much thromboplastic activity in any one of the phosphatide fractions. It appears that the complex of phosphatides, lipids, and protein making up the unit lipoprotein molecule must be required in the coagulation process, since the whole lipoprotein complex was of the order of a thousandfold more active than

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lipid extracted from it. More recently Maltaner and associates (Ann. Rept. Division Laboratories and Research, N. Y. State Dept. Health, 11-13, 1952) reported that of the phosphatides only phosphatidyl serine shows a strong thromboplastic activity. Much work remains to be done in clearing up this problem.

Overman and Wright (J. Biol. Chem., 174, 759, 1948) reported the isolation of an anticoagulant from beef brain and rabbit lung thromboplastins, and from human plasma and soy bean phosphatides, which was an inositol phosphatide. ~~Kielley and~~

VI. The phosphatides appear to be important as components of enzyme systems.

Swanson and Mitchell (Federation Proc., 11, 296, 1952) found considerable amounts of phosphatide in a highly purified preparation of liver nuclei adenosine triphosphatase (ATPase). Earlier, Kielley and Meyerhof (J. Biol. Chem., 183, 391, 1950) described a preparation of muscle ATPase containing much phosphatide. They found that the enzyme was inactivated by the action of phospholipase D of Clostridium welchi, which splits off phosphorylcholine (choline phosphate) from lecithins (phosphatidyl cholines). This work indicates that a lecithin component of the enzyme is essential for activity.

Kutscher and Sieg (Naturwiss., 37, 451, 1950) claim that choline pyrophosphate is present in both acid and alkaline phosphatases.

VII. Phosphatides or their derivatives are necessary for fat metabolism in the liver.

As previously outlined in the discussion of fatty livers, normal fat metabolism in the liver is associated with a rapid metabolic turnover of phosphatides, and any of many conditions interfering with phosphatide metabolism in the liver decreases the capacity of the liver to metabolize fats.

VIII. Phosphatides or their derivatives are necessary for oxidation of fatty acids in liver and other tissues.

Artom (J. Biol. Chem., 205, 101, 1953; 213, 681, 1955) studied the oxidation of C^{14} labeled stearic and palmitic acids by liver slices, homogenates, and washed particles of rats maintained on various diets by following the C^{14} in the CO_2 evolved and also in the acetoacetate formed. Liver preparations from rats on low protein diets plus guanidoacetic acid (deficient phosphatide formation in the liver) produced less isotopic CO_2 than did the rats on the stock diet (liver phosphatides

normal). When the low protein-guanidoacetic acid diet was supplemented with choline, or a massive dose of choline was injected shortly before the rats were killed, the ability of the tissue to produce $C^{14}O_2$ at a high rate was restored. Addition of choline, betaine aldehyde, betaine, or phosphorylcholine in vitro did not stimulate the production of $C^{14}O_2$.

This work indicates that the action of choline in preventing fatty livers, to a large degree, may result from the increased rate of fatty acid oxidation in the liver due to the action of a substance or substances formed from choline in vivo.

In similar experiments (second reference above) Artom found that homogenates of rat liver, heart, and kidney showed definite increases in the rate of oxidizing fatty acids as a result of both choline supplement in the diet and injection before sacrifice of the animals. In some instances oxidation in brain homogenates also was increased.

Thus it appears that phosphatides play an essential role specifically in promoting fatty acid oxidation not only in the liver, but also in other tissues.

In a paper just published Rodbell and Hanahan (J. Biol. Chem., 214, 595, 1955) report studies on the effect of lecithins (phosphatidyl cholines) upon oxygen uptake by the mitochondria of rat and guinea pig liver, and also the oxidation of palmitic acid and tripalmitin (triglyceride) labeled with C^{14}. These workers found that lecithin and lysolecithin (α'- fatty acid removed from lecithin) increased the rate of oxygen uptake by rat liver mitochondria. With guinea pig liver mitochondria, lysolecithin, glycerylphosphorylcholine, glycerophosphate plus choline, and phosphorylcholine, but not lecithin, caused a greater stimulation of oxygen uptake than would be required for the complete oxidation of these compounds. Phosphorylcholine was most effective in this stimulation, but it did not increase the oxidation of C^{14} palmitic acid or C^{14} tripalmitin. Lysolecithin and glycerylphosphorylcholine, in the presence coenzyme A, stimulated the oxidation of palmitic acid, the glycerylphorylcholine (lecithin with the two fatty acid groups removed) being the more effective.

Since the mitochondria have been shown to be primary sites of oxidation in the cell, these results appear to be of prime importance in showing that phosphatides or their derivatives are intimately concerned with tissue oxidations.

Since the cell mitochondria contain much phosphatides, and in view of all of the above work, it may be postulated that the oxidative enzymes of mitochondria contain lecithin or other phosphatides or their derivatives as integral and essential constituents.

IX. The phosphatides appear to be widely involved in immune reactions as attested by the large number of publications on the subject. A review of this field is given in "The Phosphatides", by Wittcoff, pp. 447-451, Reinhold, New York, 1951.

Horsfall and Goodner (J. Bact., 35, 207, 1938; J. Immunol., 31, 135, 1936) found that Type I antipneumococcus sera extracted with lipid solvent to a large degree lose their flocculating capacity, but the addition of a small amount of lecithin to horse sera or of cephalin to rabbit sera, restores the ability to flocculate Type I pneumococcus.

Hazato (Z. Immunitätsforsch., 89, 1, 1936) reported that both lecithin and cholesterol increase the hapten activity of alcoholic extracts of tissues.

The phosphatide fraction of the tubercle bacillus, which contains phthioic acid as a constituent, is responsible for tubercle formation in the host. This has been referred to in earlier discussion.

Chargaff and Schaefer (Ann. Inst. Pasteur, 54, 708, 1935) concluded that all of the antigenic activity resides in the phosphatide fraction of Bacillus Calmette-Guerin. It is probable that a protein was associated with the phosphatide (E.S.W.).

The antigens used for the detection of syphilis are believed to consist largely of mixtures of phosphatides and cholesterol in varying proportions. Cardiolipin, a complex phosphatide from heart, has been shown to fix complement with syphilitic sera if lecithin or cholesterol is present (J. Biol. Chem., 143, 247, 1942; 153, 343, 1944; 161, 71, 1945). Mixtures of pure lecithin and cholesterol have been

found to be an artificial antigen specific for the Wasserman test (Z. Microbiol. Epidemiol. Immunitätsforsch. (U.S.S.R.), No. 11, 29, 1940; Chemical Abstracts, 36, 2909, 1942).

Brown and Kolmer (J. Biol. Chem., 137, 525, 1941) found the floccules formed in the Kahn test to contain what appeared to be lecithin and cephalin fractions.

Hanger (Trans. Assoc. Am. Physicians, 53, 148, 1938; J. Clin. Invest., 18, 261, 1939) observed that saline cephalin emulsions are flocculated by serum of individuals suffering from degenerative or destructive liver diseases.

X. The phosphatides appear to possess some antioxidant properties in the oxidation of fats and oils. Olcott and Mattill (Science, 100, 226, 1944; Oil and Soap, 13, 98, 1936) found the antioxidant property in the cephalin and inositol phosphatides.

XI. Since some of the phosphatides supply so-called "essential fatty acids" (linoleic, linolenic~~, and arachidonic~~), the inclusion of such phosphatides in the diet should provide an excellent source of these acids under conditions of a dietary deficiency. This would be expected to develop particularly in cases leading to steatorrhea, and other cases in which intestinal absorption of fats is poor and the diet is deficient in essential fatty acids. Soy bean phosphatides contain much of the essential fatty acids, and also have a marked effect in promoting fat absorption. The above true if the phosphatide fatty acids are well absorbed.

Brown and associates (J. Nutrition, 16, 511, 1938) maintained an adult male for six months on a diet which supplied only 0.03 gram of fat per kilogram of body weight per day. Under these conditions no harmful effects were noted. However, the diet was low enough in fats so that when rats were placed upon it, they developed typical unsaturated fatty acid deficiency symptoms. In the man the iodine number of the total serum fatty acids was decreased from an average of 123 before the experiment to 93 during the fat restriction, indicating a marked drop in unsaturated fatty acids. Of especial interest was the fact that the serum levels of linoleic

and arachidonic acids (essential fatty acids) fell to around one-half of the original values, showing that the human like the rat cannot synthesize these unsaturated acids and that they should be included in the diet. It is probable that the reason the human showed no symptoms was that his tissue reserves of these "essential fatty acids" were sufficient for the experimental period.

Hansen (Am. J. Diseases Children, 53, 933, 1939) found the fatty acids of the serum lipids of infants and children with eczema to contain less unsaturated fatty acids than normal, and this did not appear due to previous diet or infection. The oral administration of large doses of an oil rich in unsaturated fatty acids raised the unsaturated fatty acids of the serum, and improved the clinical condition.

Bodman and Felix in England during world war II (Med. Press and Circ., 209, 331, 1943) found ointments and emulsions for oral use high in unsaturated fatty acids to be valuable in treating certain types of dermatitis.

The above list of functions and actions of the phosphatides shows that they are important in a large variety of biological relations. They serve not only as essential constituents of cells but also are involved in many physiological processes.

If soy bean phosphatides (commercial soy bean lecithin) are efficiently broken down and absorbed into the blood stream they should supply to the body:

1. Choline, which is classed with the water-soluble vitamins, and which has a number of important functions.

2. A large amount of the so-called "essential fatty acids".

3. Inorganic phosphate, which is utilized in the structure of teeth and bones, and many other constituents of the body which are vitally concerned in the metabolism of all foods. The phosphorus of soy bean phosphatides has been shown to be efficiently utilized in bone formation, which means that the phosphate group of the phosphatides is efficiently split off and utilized generally.

4. Inositol, which also is a water-soluble vitamin, and is a necessary

component of tissue constituents.

5. Ethanolamine, which the body uses in making its own cephalins (phosphatidyl ethanolamines) and choline.

6. Glycerol, which contributes to the supply of glycerol from the fats and from synthesis in the body.

Thus, all components of the phosphatides are physiological substances.

Of especial interest is the established fact that commercial soy bean lecithin (phosphatides) markedly improves fat digestion and absorption, and the absorption of vitamin A. It is probable that this is true of other fat soluble vitamins (D, E, K) also, and it may be that it would promote the digestion and absorption of proteins in a mixed diet where they are present with fats (as is generally the case) due to its emulsifying action on the mixture. This might be of considerable importance in intestinal and pancreatic diseases associated with poor absorption.

The fact that choline and other derivatives of phosphatides have been shown to stimulate oxidative processes in the liver and other tissues indicates the probable value of soy bean phosphatides in improving the level of tissue metabolism. This would be especially important in older people where metabolic processes are decreased.

Soy bean lecithin should be particularly valuable for debilitated geriatric patients in improving intestinal absorption, and in stimulating metabolism and providing all of the substances enumerated above if it is well digested and absorbed. That its phosphate group is efficiently split off and utilized has been demonstrated.

The mass action effect of flooding the body with all of the digestion products of phosphatides at regular intervals so that they may be used efficiently for the formation of the many phosphatides of the body may be of particular importance in conditions in which the metabolic processes are decreased. This would be analogous to giving to patients doses of well balanced mixtures of amino acids, all of which being present in increased amounts at the same time markedly increases the efficiency of tissue protein synthesis.

Suggestions Relative to Research on Soy Bean Lecithin

The following comments are based purely upon findings in the literature so far as I have made them, because I have not been an active worker in this field.

I. Study of Intestinal Absorption of Soy Bean Lecithin

This is of primary importance. While other forms of lecithin (true lecithin or phosphatidyl cholines) have been shown to be absorbed (noted in our review) little information exists as to the absorption of soy bean lecithin, except that the phosphate component is efficiently split off and utilized. While Dr. Bauer's project, which I assume he plans on human subjects, should give some information relative to fatty acid absorption, if these acids are labeled with I^{131} they will not then be physiological (though I will bet they will be absorbed). Also, since the phosphatide fatty acids may be split off in the intestine and recombined in the intestinal wall with glycerol as triglyceride and pass into the lymph and blood stream as such, following them will not necessarily give the picture as to what happens to the phosphatides as a whole. The phosphatide molecules from which the fatty acids are split will also be recombined with other fatty acids in passing through the intestine wall to complicate the situation. Labeling the phosphate group with P^{32} and studying its distribution is of questionable value, since soy bean lecithin phosphate already has been shown to be efficiently split off and utilized.

If Glidden could grow some soy beans in the presence of the N^{15} isotope so the lecithin would be labeled with it, this material would be very valuable to use in following the absorption and metabolic fates of both the choline and ethanolamine components in animals. It could be used in humans and the increase in N^{15} choline and ethanolamine components of blood followed.

If Glidden would grow the soy beans in the presence of $C^{14}O_2$ then all of the organic components of the lecithin, fatty acids, choline, ethanolamine, inositol

and glycerol would be labeled and their absorption and metabolic fates in animals (but not humans) could be followed. It would be a good experiment if you could get it done.

I would like to suggest an absorption experiment on rats of a more comprehensive type which I believe would be of value. The technique should be that of Cori in studying the absorption of sugars by rats (J. Biol. Chem., 66, 693, 1925). The experiment should be conducted as follows:

Normal, healthy rats (120-180 grams) are fasted for 48 hours in cages constructed to remove the feces as excreted so the rat cannot eat them. Water is given ad lib. An emulsion of the phosphatides is given by stomach tube, and after a period of hours, probably 6-8, the animals are killed and the entire digestive tract removed, and thoroughly washed out with physiological saline to remove all contents, which are saved for analysis.

The total contents of fatty acids, phosphate, choline, ethanolamine, glycerol, and inositol of the phosphatide emulsion given to the rats are determined, and also these values are determined on the intestinal contents after the period of absorption. From the differences between the values for these substances administered and remaining in the intestinal contents after absorption, the amount of each component of the soy bean lecithin absorbed may be calculated.

It would be necessary to run controls in which the above substances are determined in the gastrointestinal contents of 48-hour fasted rats (plus 6 to 8 hours of absorption time allowed for the lecithin animals) not given the lecithin.

In a similar way the effect of the lecithin emulsion could be used to determine its effect upon the absorption of fats, proteins, calcium and other minerals, and vitamins, except in this case the analyses would be limited to those concerned with the added substance. In each case a lecithin emulsion containing the added substance could be prepared for stomach tubing. This paragraph is an interpolation, "discussed".

This type of experiment should give the answer as to the completeness of

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CONTRIBUTED PAPERS

METHOD FOR DIFFERENTIATING BETWEEN EGG LECITHIN AND SOYBEAN LECITHIN IN MACARONI AND NOODLE PRODUCTS*

By James J. Winston and Benjamin R. Jacobs (National Macaroni Manufacturers Association, New York, N. Y.)

Lecithin, as found in eggs and in soybeans, is very similar both in composition and properties. Investigators in the past have had great difficulty in differentiating between these two forms and in detecting one in the presence of the other (1). Attempts to detect soybean lecithin have been based largely on chemical constants such as the phosphorus and nitrogen percentages and the ratio between the two. (2)

Lecithin, a phospholipid, is a compound of phosphoric acid, glycerol, higher fatty acids, and choline. Winterstein and Hiestind (1) in 1906 found that plant phosphatides on hydrolysis yield not only the usual decomposition products of lecithin but sugar as well. Consequently, it was found that the phosphorus content of lecithin derived from eggs was higher than the phosphorus content of lecithin derived from plant life. The same was true of the other constituents, such as nitrogen and choline. Some analysts have made these facts the basis for distinguishing between the two types. However, these differences are too small to enable the analyst to definitely detect the presence of one type when mixed with the other.

Since soybean lecithin is finding its way into use in many food products, *e.g.*, candy, oleomargarine, shortening agents, and (experimentally) noodle products, it was deemed sufficiently important to investigate methods for its detection in order to prevent the simulation of eggs in certain products, principally noodles.

Prior to 1941, the method for the determination of egg solids in noodle products was based on the lipoid phosphoric acid content (3). The substitution of soybean lecithin, because of its high lipoid phosphoric acid, would therefore be confusing in this determination and would vitiate the analysis. In fact, the presence of soybean lecithin in an egg noodle makes it very difficult to arrive at the proper result for egg solids by any method now in use. The method of E. O. Haenni (4), which depended on the cholesterol content is also vitiated in the presence of soybean lecithin (5).

Soybean lecithin has the property of fluorescing blue and exhibits this very strongly when subjected to ultra-violet light (6), whereas lecithin derived from flour and egg products shows this property very feebly. The following method was built on this principal.

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* Presented at the 59th Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 25 and 26, 1944.

METHOD OF ANALYSIS

APPARATUS AND REAGENTS

Pfaltz and Bauer Fluorophotometer, Model B, with accessories. Filter set #630 consisting of one ultra-violet filter with a peak of 3700 Angstrom units. A double filter placed between cuvette and photo-cell with a peak of 4600 Angstrom units. These filters are identical to those used in the Thiochrome determination (7). The fluorophotometer was so standardized with quinine sulfate, one microgram per ml. in 0.1 *N* sulfuric acid, as to give a fluorescence of 50 divisions on the mirror galvanometer. This constituted the setting point in all the following experiments.

Wide stoppered glass bottles.* 95 percent ethyl alcohol, acetone C.P., naphtha, quinine sulfate U.S.P., anhydrous sodium sulfate C.P.

PROCEDURE FOR FLOURS, SEMOLINAS, MACARONI, AND NOODLE PRODUCTS

To a 5-gram sample, in a wide stoppered bottle, add 7 ml of 95 percent alcohol followed by addition of ½ gram of anhydrous sodium sulfate. After soaking ½ hour, add 93 ml of naphtha, shake the bottle well, and permit to stand in the dark overnight. Pipet the clear solution into a cuvette and examine for fluorescence; make a blank correction on all the readings.

PROCEDURE FOR YOLKS AND WHOLE EGGS

Place in wide-stoppered bottle suitable amount of egg product (previously weighed out on a strip of aluminum) and add 7 ml of acetone. Break up and disintegrate the egg by means of flattened end of stirring rod. Add ½ gram of anhydrous sodium sulfate and soak for ½ hour. Add 93 ml of naphtha, shake bottle well, and allow to stand in the dark overnight. Pipet the clear solution in a cuvette and determine its fluorescence, as above.

EXPERIMENTAL RESULTS

The soybean lecithin** used in this work contained about 60.0 percent phospholipids and 40.0 percent vegetable oil, which is typical of the average composition of commercial soybean lecithin. Different amounts of this product were dissolved in alcohol-naphtha solution, 7 ml of 95 percent ethyl alcohol and 93 ml of naphtha. The fluorescence of the different quantities of lecithin was obtained on the instrument and recorded in terms of scale divisions in order to determine whether there existed any relationship between the concentration and the fluorescence. Examination of a number of different commercial samples of soybean lecithin for fluorescence indicates that the variation that can be expected from these figures is about 8–10 percent, depending upon the amount of phospholipids present. (Commercial lecithin has a phospholipid content ranging from 55.0 to 65.0 percent which is mixed with a vegetable oil carrier to the extent of 35.0 to 45.0 percent.)

These results, plotted in Fig. 1, show that there exists a relationship between concentration and fluorescence, and that up to a concentration of 212 milligrams, the Lambert-Beer Law holds (8). Inasmuch as egg noodles

* Caution: Do not use rubber stoppers as this will impart fluorescence to the solution.
** Obtained from American Lecithin Company, Elmhurst, L. I., N. Y.

cannot be expected to contain more than 3 percent lecithin, owing to its shortening effect, this fluorescence-concentration relationship can be considered satisfactory for the determination of added soybean lecithin in noodle and macaroni products.

Samples of the usual farinaceous ingredients, flour, farina, and semolina, were examined for fluorescence by using the extraction method for carotenoids as advocated by Ferrari and Bailey (9), and Munsey (10).

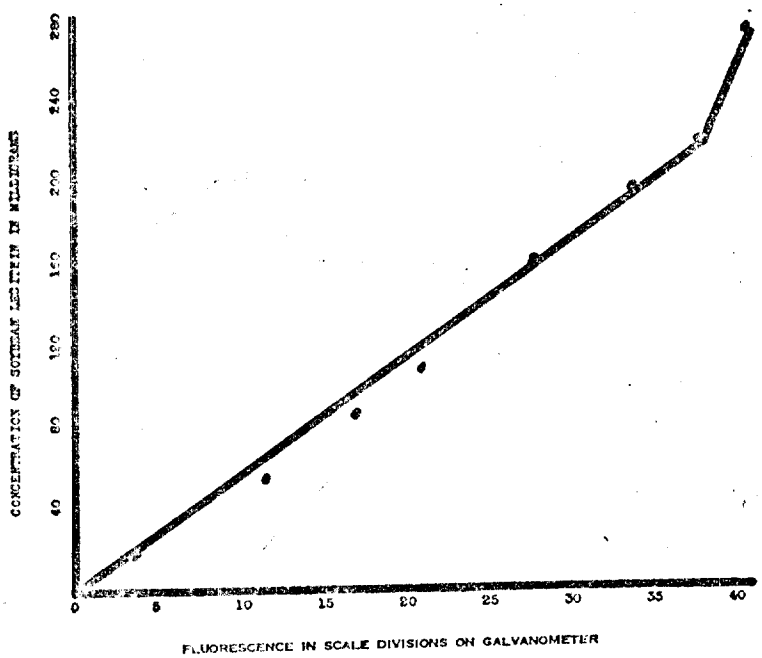

FIG. 1.—Concentration of soybean lecithin in milligrams.

A 5-gram sample was extracted with the alcohol naphtha reagent (7:93) for 24-hours in a wide stoppered bottle and the clear solution was pipetted into a cuvette and examined for fluorescence. The reagents used were examined for fluorescence and a blank correction was made on all readings. The results as indicated in Table 2 show that these ingredients exhibit a very small degree of fluorescence. This table shows typical examples taken from a much larger number. More than one hundred individual readings for fluorescence were made.

Egg products, consisting of frozen egg yolk and whole eggs, were likewise examined for fluorescence. Representative samples from a large number tested have been tabulated in Table 3. It will be noted that in this range the fluorescence of the yolks varied from a minimum of 1.0 to

TABLE 1.—*Concentrations of soybean lecithin*

CONCENTRATION IN MILLIGRAMS	FLUORESCENCE IN SCALE DIVISIONS
1.0	0.2
9.0	2.0
18.8	4.0
51.0	11.5
82.4	17.0
104.1	21.0
153.0	28.0
190.0	34.0
212.0	38.0
268.0	41.0

a maximum of 3.0 but it is interesting to see that the concentration of the yolk did not have any significant effect on the fluorescence. Whole eggs in general showed less fluorescence, with a maximum of 1.5 scale divisions, whereas an amount of soybean lecithin as small as 18.8 milligrams gave a scale deflection of 4.0 divisions.

The following procedure was used to determine which one of the components of soybean lecithin was the cause of the high fluorescence.

A sample of soybean lecithin was treated with acetone (11) and the filtrate, consisting of the vegetable oils, was filtered through a pledget of

TABLE 2.—*Typical examples of fluorescence in flour, etc.*

PRODUCT	WT. OF SAMPLE EXTRACTED IN GMS.	FLUORESCENCE IN SCALE DIVISIONS
Semolina	5.0	1.0
Semolina	5.0	0.5
Semolina	5.0	0.5
Semolina	5.0	1.0
Semolina	5.0	0.5
Farinas	5.0	0.5
Farinas	5.0	0.0
Farinas	5.0	0.0
Durum Flours	5.0	1.0
Durum Flours	5.0	1.0
Durum Flours	5.0	0.5
Durum Flours	5.0	0.5
Durum Flours	5.0	0.5
Durum Flours	5.0	0.5
Durum Flours	5.0	0.5
Durum Flours	5.0	1.0
Durum Flours	5.0	1.0
Durum Flours	5.0	1.0

TABLE 3.—*Samples of fluorescence in eggs*

PRODUCT	WT. OF SAMPLE EXTRACTED IN MGMS.	FLUORESCENCE IN SCALE DIVISIONS
Egg Yolks	853.6	1.5
Egg Yolks	807.2	2.5
Egg Yolks	729.6	3.0
Egg Yolks	534.0	3.0
Egg Yolks	556.8	2.5
Egg Yolks	637.8	3.0
Egg Yolks	800.0	1.0
Egg Yolks	654.4	1.5
Egg Yolks	601.8	1.5
Egg Yolks	843.0	1.5
Whole Eggs	915.8	0.5
Whole Eggs	883.0	1.0
Whole Eggs	843.0	1.5
Whole Eggs	599.0	1.0
Whole Eggs	980.0	1.5
Whole Eggs	670.0	1.0
Whole Eggs	779.4	1.5
Whole Eggs	435.5	0.5
Whole Eggs	500.0	1.0
Whole Eggs	779.5	1.0

cotton into a weighed aluminum dish. The residue on the filter was then treated with ethyl ether which dissolves the lecithin, and this extract was collected into another weighed aluminum dish. Both dishes were placed on the water bath and evaporated to dryness, dried in a water oven at 100°C. for one hour, and finally weighed. The two constituents of the above soybean lecithin were then examined for fluorescence in the usual manner. It will be noted from Table 4 that the phospholipid fraction is the cause of the fluorescence.

TABLE 4.— *Fluorescence of vegetable oil compared with lecithin*

WEIGHT OF SAMPLE	WEIGHT OF VEGETABLE OIL	PERCENT	WEIGHT OF LECITHIN	PERCENT	FLUORESCENCE, SCALE DIVISIONS	
					VEGETABLE OIL	LECITHIN
grams 0.7430	*grams* 0.2006	27.0	*grams* 0.5356	72.1	5.0	55.0

Several different samples of macaroni products were manufactured under strict supervision, and different quantities of soybean lecithin were incorporated. The same mixer, kneader, and press were used for each product, and they were all subjected to the same drying conditions.

These finished products were examined for fluorescence by extracting

a 5 gram, finely ground sample, with the usual alcohol naphtha reagent to make a total volume of 100 ml. It will be noted from Table 5 that the presence of a small amount of soybean lecithin was sufficient to give an abnormal degree of fluorescence. Plotting these values on Fig. 2 shows that the intensity of the fluorescence is a function of the concentration of the

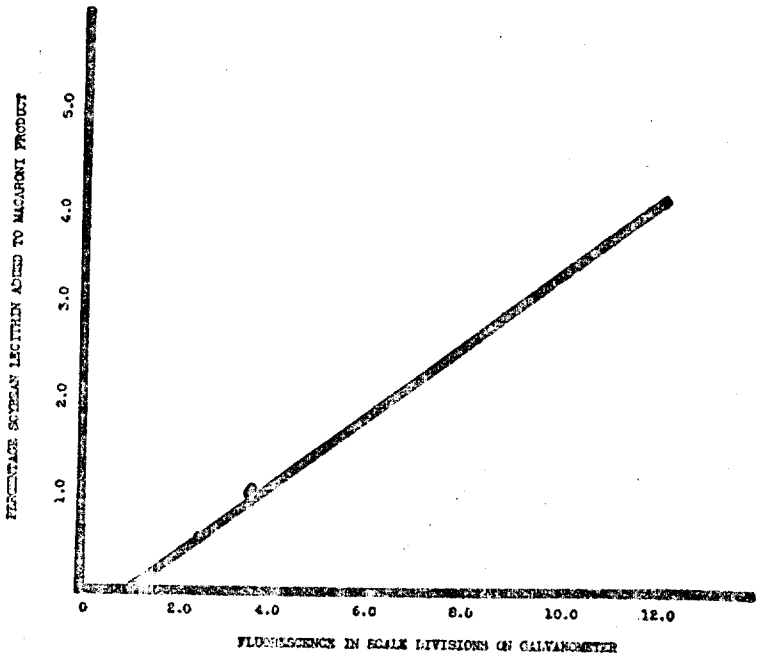

FIG. 2.—Percentage soybean lecithin added to macaroni product.

soybean lecithin and that the galvanometer deflection is generally in linear relationship to the concentration of the soybean lecithin.

TABLE 5.—*Percentage soybean lecithin added to macaroni products*

ADDED SOYBEAN LECITHIN	FLUORESCENCE IN SCALE DIVISIONS
percent	
0.0	1.0
0.5	2.5
1.0	3.5
2.0	6.5
3.5	10.5

A large number of macaroni products including spaghetti, macaroni, and elbows, manufactured in different macaroni factories, were subjected

TABLE 6.—*Fluorescence in macaroni products*

PRODUCT	FLUORESCENCE SCALE DIVISIONS
Macaroni	1.0
Macaroni	0.5
Macaroni	1.0
Macaroni	1.0
Macaroni	0.5
Spaghetti	0.5
Spaghetti	0.5
Spaghetti	1.0
Spaghetti	1.0
Spaghetti	0.5
Elbows	1.0
Elbows	1.0
Elbows	1.0
Elbows	0.5
Elbows	0.5

to the fluorescence test. As will be noted from Table 6, the fluorescence obtained in all cases was not over 1.0 scale division, whereas the presence of 0.5 percent soybean lecithin was sufficient to cause an abnormal fluorescence of 2.5 divisions.

Egg noodles containing 5.5 percent egg solids, as yolk, were likewise manufactured under supervision using the same mixer, kneader, and cutter, and the same drying process. Different quantities of soybean lecithin ranging from 0.5 to 2.0 percent, were added. The dried products were examined for fluorescence using a 5-gram, finely ground sample in each instance. The results, as indicated in Table 7 and Fig. 3, show that the fluorescence is practically proportional to the amount of lecithin present in the sample.

The addition of 0.5 percent soybean lecithin to an egg noodle can therefore be detected, owing to its significant fluorescence as compared to the feeble fluorescence exhibited by a normal egg noodle made solely from eggs.

TABLE 7.—*Percentage soybean lecithin added to egg noodles*

ADDED SOYBEAN LECITHIN	FLUORESCENCE IN SCALE DIVISIONS
percent	
0.0	1.0
0.5	4.5
1.2	11.5
2.0	19.5

Two hundred samples of egg noodles containing 5.5 percent egg solids have been examined in the past year. A statistical analysis indicates that the maximum fluorescence was 2.5 scale divisions, the minimum 1.0 scale divisions, the average 1.6 scale divisions, and the standard deviation 0.45 scale division. In Table 8 are the results of typical egg noodles, some made with yolk, some with whole egg, and some with dried eggs. The maximum

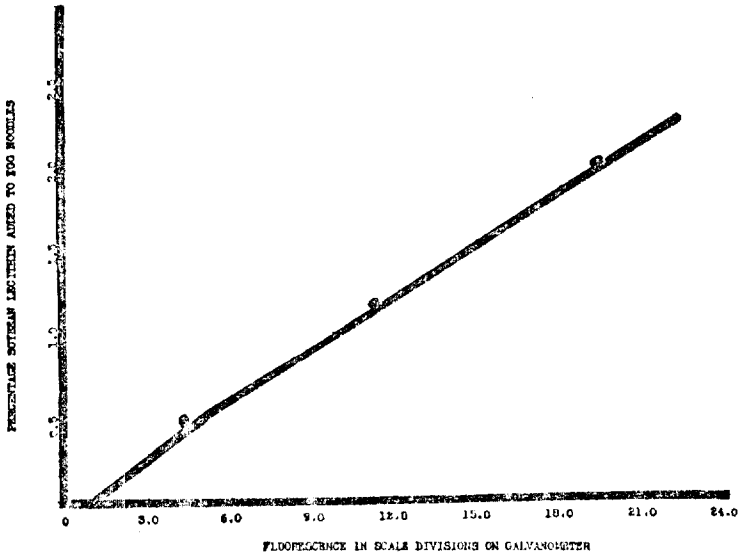

FIG. 3.—Percentage soybean lecithin added to egg noodles.

fluorescence in no case exceeded 2.5 scale divisions, whereas the addition of 0.5 percent soybean lecithin was sufficient to increase the fluorescence by 80-100 percent. The addition of 0.5 percent commercial soybean lecithin to a noodle product would correspond to 1.4 percent added egg solids as yolk, since this product has on the average 60 percent phospholipids.

TABLE 8.—*Maximum fluorescence in egg noodles*

NUMBER	FLUORESCENCE	NUMBER	FLUORESCENCE
1	1.0	11	2.5
2	1.0	12	2.0
3	1.5	13	1.0
4	1.0	14	1.5
5	1.2	15	1.0
6	1.4	16	1.5
7	1.0	17	1.0
8	1.2	18	1.0
9	1.6	19	1.6
10	1.0	20	1.0

The present Standards of Identity by the Food and Drug Administration include a macaroni product containing 12.5 percent soy flour. Experiments were therefore conducted to ascertain if the presence of this amount of soy flour, with its high lecithin content, would have any significant effect on the fluorescence of the finished product. Four different macaroni products were manufactured, each containing 85 percent durum flour and 15 percent soy flour. The finished products were examined in the usual manner using a 5-gram, finely ground sample.

TABLE 9.—*Maximum fluorescence in soy macaroni products*

TYPE SOY FLOUR USED	FLUORESCENCE IN SCALE DIVISIONS
1—Extracted Soy Flour, Fat content—1.0%	2.0
2—Extracted Soy Flour, Fat content—1.0%	2.0
3—Medium Fat—7.0%	2.0
4—High Fat—22.0%	2.5

It will therefore be noted, from Table 9, that the presence of 15 percent soy flour in a macaroni product will increase the fluorescence somewhat. However, a correction factor can be applied to a macaroni product, to yield the fluorescence due solely to the product exclusive of the soy flour used.

SUMMARY AND CONCLUSIONS

A need for a method of differentiating between soybean lecithin and egg lecithin has been demonstrated.

The strong fluorescence of soybean lecithin, when subjected to the ultra-violet light, has been proved to be an adequate basis for detecting the addition of soybean lecithin to macaroni and noodle products.

It has been demonstrated that the farinaceous ingredients and egg products show the property of fluorescence to only a very small extent, and that the addition of 15 percent soy flour will not tend to interfere with the detection of soybean lecithin.

The fluorescence obtained by the use of added soybean lecithin is practically proportional to the concentration used, and hence may serve as a means of estimating the amount added to a macaroni or noodle product.

A definite method for determining the addition to macaroni and noodle products of quantities of soybean lecithin as small as 0.5 percent (in most cases) has been presented and proposed.

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ESTROGEN IMPLANTS IN DOGS

A Preliminary Report

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THIS was a study to test the effects of estrogen implants (alpha-estradiol) in dogs. The main interest centered about the oral tissues, although other organs were observed as well. Gross and microscopic examinations were made, and complete blood counts and pertinent blood chemical analyses were included.

Two studies dealing with the effects of estrogen in dogs are reported in the literature: Crafts (1941) injected daily doses of 5 mg. alpha or beta estradiol and found a marked neutrophilia followed by a neutropenia, a decrease in young neutrophiles, an anemia, and a decrease in hemoglobin. Tyslowitz and Dingemanse (1941) injected daily 5 mg. estrogen into dogs and found a fall in nearly all the blood elements. In many there was a picture of almost complete absence of granulocytes in the peripheral blood with similar decreases in the bone marrow, and necrosis of mucous membranes and skin, described in man as agranulocytosis. Skin symptoms were edema around the mouth and sex skin, loss of hair, necrosis between toes, poor healing of wounds, and hemorrhages under the skin and mucous membranes of the alimentary canal or the urogenital tract.

Our study differed from both of these in that estrogen was implanted subcutaneously and the experiments were of longer duration. While some of our findings were similar, new factors were introduced, and symptoms not reported in either of the two studies mentioned above were observed.

Twenty-one females (mostly mongrel fox terriers of varying ages), weighing from six to sixteen pounds, comprised our group. They were divided into four categories as follows:

Group 1.—Thirteen dogs received implants of alpha-estradiol pellets subcutaneously.

Group 2.—Three dogs received the estrogen implants and in addition were given 3 Gm. soybean lecithin daily incorporated into their diet.

Group 3.—Three dogs received estrogen implants, only, for several months; then 3 Gm. lecithin were added to their daily diets, twenty-eight days before

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the termination of the experiment in two instances and twelve days in one instance.

Group 4.—Two controls, living under conditions identical with the rest of the group but without estrogen implants or lecithin.

METHODS

Routine procedures consisted of: pre-experimental weight; x-rays of the jaws and teeth, and the extremities; biopsies of the gingivae; taking of fasting blood for complete blood count; sedimentation rate; tests for blood sugar, phosphatase, phosphate, cholesterol, vitamins A and C; and collection of urine for assays of thiamine, riboflavin, and nicotinic acid. The estrogen was implanted after these preliminary procedures which were subsequently repeated at intervals of about three months.

In group 1, five animals died, four were sacrificed because they were on the verge of death, and four were sacrificed at the termination of the experiment. The average duration of the experiment in this group was thirty-two and four-tenths weeks, the animals living the longest having been under observation for seventy-two weeks, the shortest span having been eight weeks. A deviation involved two animals receiving implants of estrogen at the beginning of the study without renewal for over a year.

In group 2, two dogs died and one was sacrificed. The average duration of experimental procedures was twenty-six and six-tenths weeks, the variation being from twenty-three to thirty-two weeks.

In group 3, one dog died and two were sacrificed. The average survival was twenty-four and seven-tenths weeks, with variations from twenty-one to thirty weeks.

In group 4, both animals were sacrificed terminally, one after twenty-seven weeks, the other after twenty-eight weeks.

Prior to sacrifice, the routine clinical and laboratory procedures previously described were again repeated; and at autopsy the following tissues were reserved for histologic examination: gingivae, mucous membranes, tongue, skin, uterus, vagina, ovaries, stomach, intestine, kidney, liver, spleen, heart, and lung. The thyroids and adrenals were studied early in the investigation, but because they showed no apparent histologic changes, the examinations were not continued. The jaws were prepared for sectioning as was a piece from one of the long bones.

Gross observations included search for changes in the gingivae and skin.

RESULTS

Weight: (Table I).

Group 1.—Ten of the thirteen dogs showed weight gain, terminally. Of these, five were immature when first examined. One lost, and two weighed about the same at the end as at the onset. All weighed more at midpoints in the experimental period than at the end. The greatest gain was six pounds. Most of the animals showed a variation of only one to two pounds.

Group 2.—Terminally, one dog showed a loss of three pounds; one an increase of one pound; and one weighed the same as at the beginning.

TABLE I. MASTER TABLE ON FINDINGS IN FEMALE DOGS IMPLANTED WITH ALPHA-ESTRADIOL PELLETS

DOG NUMBER	DIED	SACRIFICED AT POINT OF DEATH	SACRIFICED TERMINALLY	DURATION, WEEKS	IMPLANTED MG.	ABSORBED DAILY µG.	WEIGHT, POUNDS, CHANGE	BLOOD COUNTS AND HEMOGLOBIN: RED BLOOD CELLS DECREASE X10⁶	RED BLOOD CELLS PER CENT	RED BLOOD CELLS MILD	RED BLOOD CELLS MODERATE	RED BLOOD CELLS SEVERE	WHITE BLOOD CELLS DECREASE X10³	WHITE BLOOD CELLS PER CENT	WHITE BLOOD CELLS MILD	WHITE BLOOD CELLS MODERATE	WHITE BLOOD CELLS SEVERE	INITIAL RISE X10³	INITIAL RISE PER CENT	HEMOGLOBIN DECREASE	HEMOGLOBIN PER CENT	HEMOGLOBIN MILD	HEMOGLOBIN MODERATE	HEMOGLOBIN SEVERE
Group I																									
807	x			19	40	0	+6	0					0							0				
736	x			40	210	220	0	0					0							0				
686	x			22	70	0	+1	1.04	18	x			17.3	79			x			6	7			
687	x			23	70	130	−1	1.11	16	x			Incr. 2.15	17						Inc. 6	7			
732		x		33	100	0	+2	0					0							0				
683		x		8	50	160	+1	4.31	50		x		13.1	93			x			14	17	x		
709		x		55	80	200	+4	*				x	*				x							
823		x		18	40	105	+5	0					0							0				
804			x	19	90	300	+1	0					0							0				
719			x	23	100	200	+6	5.22	68			x	14.1	90			x	3.1	19	20	22	x		
689			x	18	100	200	+1	0.26	4				11.2	70			x			10	11	x		
821			x	72	40	80		0.01	0				12.6	54		x		2.1	9	10	12	x		
819			x	72	60	120	+6	0.08	1				5.9	32		x		1.5	9	18	18	x		
Group II																									
718	x			25	70	130	−3	2.1	33		x		9.6	87			x	2.4	22	24	27	x		
684			x	32	70	180		3.5	40		x		6.0	45		x		1.0	7	18	20	x		
978			x	23	70	0	+1	1.8	22	x			9.3	47		x		3.3	17	2	2			
Group III																									
688	x			23	100	300	+ .5	3.68	44		x		8.2	65			x			34	39			x
701			x	21	70	230	−2	0.49	7	x			1.0	11	x			5.2	53	18	17	x		
824			x	30	100	200		Incr. 1.06	18				6.1	49		x		39.0	310	12	13	x		
Group IV Control																									
685			x	27			+2	Incr. 0.73	1				14.2	40		x				14	16	x		
822			x	28			+1	1.21	1				2.1	14	x									

X—findings listed; O—no observation; blank—no change; *—no initial blood study, terminal blood taken only; Incr.—increase.

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Group 3.—Terminally, one dog showed an increase of one pound; one a loss of two pounds; and one weighed the same as at the beginning.

Group 4.—(Controls.) These animals showed a gain of one and two pounds, respectively, in the final weighing.

Implants of Alpha-Estradiol: (Table I).

The average total dosage over the entire period was 80 mg. All animals received implants at least twice, and some as many as five times.

Group 1.—The greatest amount implanted in one animal was 210 mg. and the least, 40 milligrams. The average absorption was about 170 μg a day. The greatest possible absorption, assuming all nonrecovered pellets to have been absorbed, was 300 μg and the smallest amount was 80 micrograms. In those instances where recovery was certain, the average quantity absorbed was approximately 130 μg, so that a more accurate estimate for the group would be between 125 and 170 μg a day.

Group 2.—Seventy milligrams were implanted in each animal. The average daily absorption was about 155 micrograms.

Group 3.—An average of 90 mg. estrogen was implanted. The largest amount was 100 mg. and the smallest was 70 mg. The estimated average amount absorbed daily was 243 micrograms.

Blood Counts: (Table I).

Group 1.—Eight of the thirteen animals had blood counts. Of these, three showed no significant red blood cell change, terminally; two showed a severe drop, one a moderate drop, and two a mild drop. The white blood cells were unaltered in one animal; in five there was a severe drop; in two there was a moderate drop. The hemoglobin was least affected. In two there was no change; in five there was a mild drop. The eighth animal did not receive the hemoglobin test.

Group 2.—Of the three animals in this group, there was a slight drop in red blood cells in one and a moderate drop in two. The white blood cells dropped severely in one and moderately in two. The hemoglobin remained unchanged in one and dropped slightly in two.

Group 3.—The red blood cells dropped moderately in one, mildly in another, and not at all in the third. In one there was a mild decrease in the white blood cells, in the second a moderate decrease, and in the third the decrease was severe. Hemoglobin was lowered slightly in two and moderately in the third.

Group 4.—(Control animals.) One showed a slight and one a moderate drop in white blood cells, and one a slightly lowered hemoglobin. Otherwise the counts were normal.

Skin: (Table II).

The skin changes consisted of alopecia; a scaly dermatitis, located chiefly on the exposed surfaces of the joints of the forelegs and hind legs, somewhat resembling psoriasis in man; scabs between the toes; and scaly sores generally over the body.

Group 1.—Seven animals had alopecia (four severe and three mild); seven were affected with the scaly dermatitis (five severe, one moderate, and one mild);

two had no skin lesions; and two developed a transitory rash during the early part of the treatment.

Group 2.—Two animals had a mild alopecia; one had a slight and one a moderate scaly dermatitis.

Group 3.—One had a moderate scaly dermatitis; the other two were negative.

Group 4.—The controls showed no skin changes.

Gingivae: (Table II).

Group 1.—Gross examination of the gingivae of twelve animals revealed an inflammatory condition in eight resembling a so-called "chronic" Vincent's infection or, in a few, a more severe necrosis characteristic of agranulocytic angina. Two showed a thickening and blanching of the gingivae typical of estrogen-induced changes. Two showed no change.

Group 2.—All had a moderate degree of inflammation of the character described in group 1.

Group 3.—The gingivae of two showed moderate inflammation; one remained unchanged.

Group 4.—The controls revealed no change.

Uterus: (Table II).

Group 1.—The uteri of eleven of the thirteen dogs were examined. Two of these cases received implants at the onset only, and survived for more than a year from that time. One of the two showed slight necrosis of the endometrium; the other showed no change. The nine dogs receiving estrogen over a long period presented one or more of the following changes: necrosis involving the endometrium, the myometrium, or perforations. In six, the ulcerations were severe; while in three they were moderate.

Group 2.—Uterine examination in two cases was negative; in one, a moderate alteration as described in group 1 was seen.

Group 3.—The animal receiving lecithin for twelve days terminally showed a moderate degree of necrosis; the two dogs receiving the lecithin for twenty-eight days showed a mild involvement of the uterus in one instance and none in the other.

Group 4.—No evidence of change was seen in the uteri of the controls.

Other Findings at Autopsy: (Table II).

Nine of eleven dogs autopsied in group 1 showed the presence of peritonitis with an exudate of foul-smelling fluid, and hemorrhage. Only one of the six animals receiving soybean lecithin showed this change. The nonconforming animal received the lecithin terminally for only twelve days. Tongue ulcers were observed in three cases.

Histologic Observations:

Gingivae.—The histologic findings of the gingivae of the entire group are incomplete. We report here in detail on one dog, these gingival findings being representative of those in the category, group 1, treated with estrogen im-

plants only. Five gingival biopsies were made in this case at approximately three-month intervals; one pre-experimentally, one at termination of the experiment, and three in between. (Figs. 1, 2, 3, 4, and 5.)

TABLE II. MASTER TABLE OF FINDINGS IN FEMALE DOGS IMPLANTED WITH ALPHA ESTRADIOL PELLETS

DOG NUMBER	SKIN						GINGIVAE						UTERUS			PERITONITIS
	ALOPECIA			SCALY DERMATITIS			PROLIFERATIVE			DEGENERATIVE			DEGENERATIVE			
	MILD	MODERATE	SEVERE	MILD	MODERATE	SEVERE	MILD	MODERATE	SEVERE	MILD	MODERATE	SEVERE	MILD	MODERATE	SEVERE	
Group I																
807			X				O			O						O
736	X					X						X			X	X
686	X			X								X			X	X
687					X					X				X		X
732						X									X	X
683							X								X	X
709			X			X		X							X	X
823										X					X	X
804						X						X			O	X
719						X				X				X		
689	X									X				X		X
821			X													
819			X							X			X			
Group II																
718	X			X						X				X		
684	X											X				
978					X							X				
Group III																
688												X		X		
701					X							X	X			
824																
Group IV Control																
685																
682																

X—findings listed; O—no observation; blank—no change.

At the end of the first three-month period, gingival changes were characterized principally by hyperplasia of the entire tissue. The nuclei in the germinal layer appeared crowded and more numerous (Figs. 2, *B* and 3, *B*); the rete pegs were somewhat pointed with splitting and proliferation forming off-shoots (Fig. 2, *B*). The axes of the prickle cells were irregular. The keratin layer appeared slightly wider and firmer than pre-experimentally although a slight degree of stripping was noticeable. The whole layer of epithelium increased about one-third in width.

The corium was hyperplastic. There was a considerable increase in the number of fibroblasts and collagenous elements. The tissue appeared firm with no evidences of edema or inflammation. The width was almost double that of the pre-experimental corium. (Figs. 2, *B* and 4, *B*.)

After six months of treatment, the gingival biopsy still showed certain hyperplastic features. Splitting and pointing of pegs were visible. However, the germinal nuclei were not as crowded, and the prickle cells had assumed

A. *B.* *C.* *D.* *E.*

Fig. 1.—Dog No. 709—gingivae. Estrogen implants only. Maximum daily absorption of 200 micrograms. Duration, fifty-five weeks. *A*, Pre-experimental. Normal epithelial layer overlying normal-appearing corium. (×20.) *B*, Experimental, three months. Hyperplastic epithelium with marked downgrowth of pegs into the corium. The corium is hyperplastic and thicker than the pre-experimental. Note increase in width of whole tissue. (×20.) *C*, Experimental, six months. Epithelium in early regressive stage. Splitting and pointing of pegs are still observable. Slight edema in the corium. (×20.) *D*, Experimental, nine months. The breakdown of the epithelium and the edema of the corium are marked. (×20.) *E*, Experimental, thirteen months. Post-mortem. Marked invasion of epithelium and corium with inflammatory exudate. Almost complete breakdown of the normal structures. (×20.)

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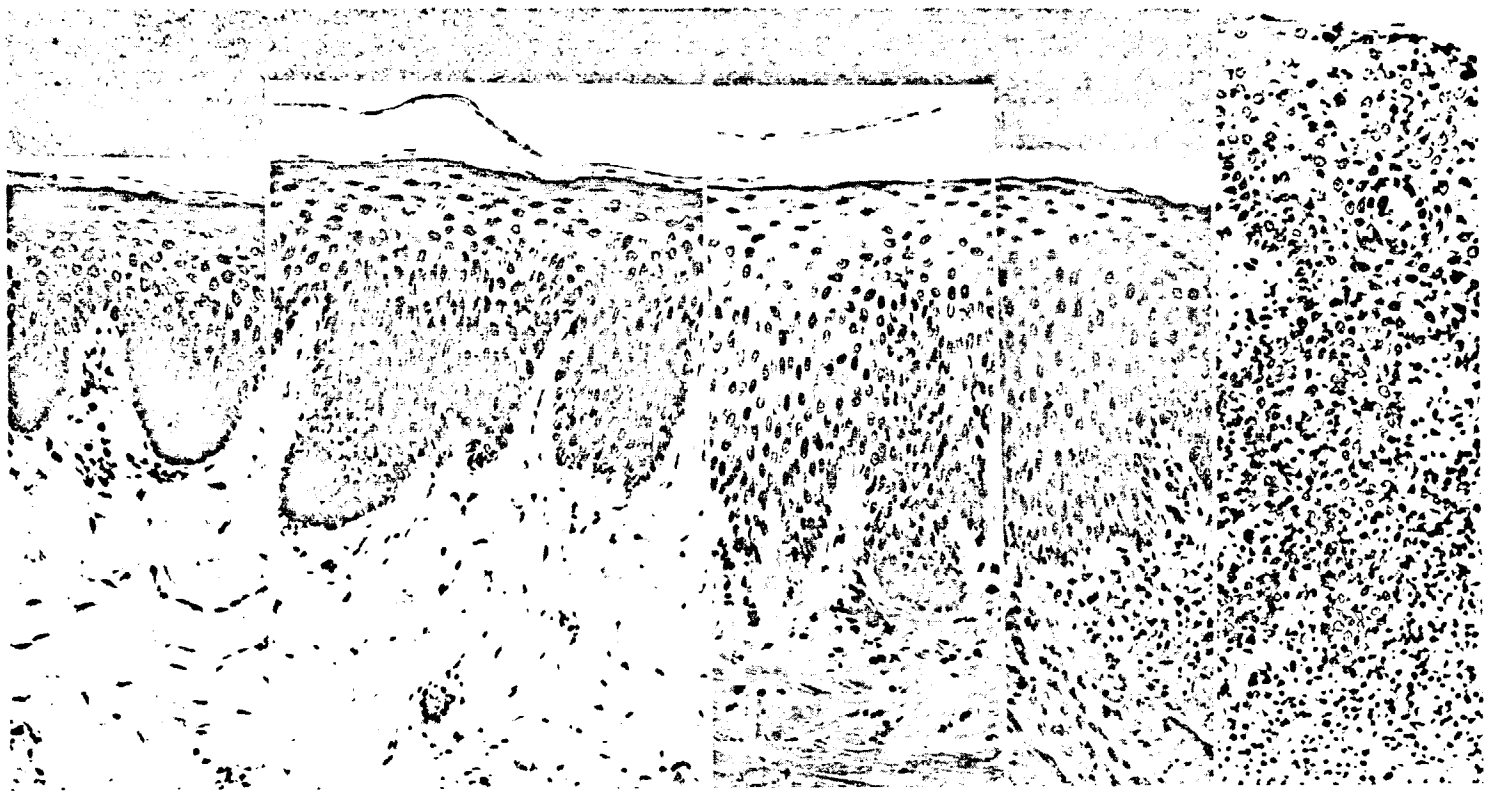

A. B. C. D. E.

Fig. 2.—Same animal as in Fig. 1. Alveolar epithelium. *A*, Normal epithelium and corium. Note keratinized surface, well-rounded pegs, normal orientation of basal and prickle cells. (×252.) *B*, Slight stripping of surface keratin. Increase and crowding of nuclei, splitting of pegs, and increase in depth are evidences of epithelial hyperplasia. The corium is more cellular, exhibiting an increase in the number of fibroblasts. (×252.) *C*, Stripping of keratin is more marked. There is a regression of the hyperplastic changes. Intercellular edema is visible in the epithelium. Edema is also present in the corium. (×252.) *D*, The keratin layer has been lost. The epithelium is no longer hyperplastic. The prickle cells have assumed a vertical arrangement. The edema in the corium is more marked. (×252.) *E*, The epithelial breakdown is extreme. The inflammatory cells have invaded the epithelium. The edema and inflammatory exudate have almost obliterated the structures of the corium. (×252.)

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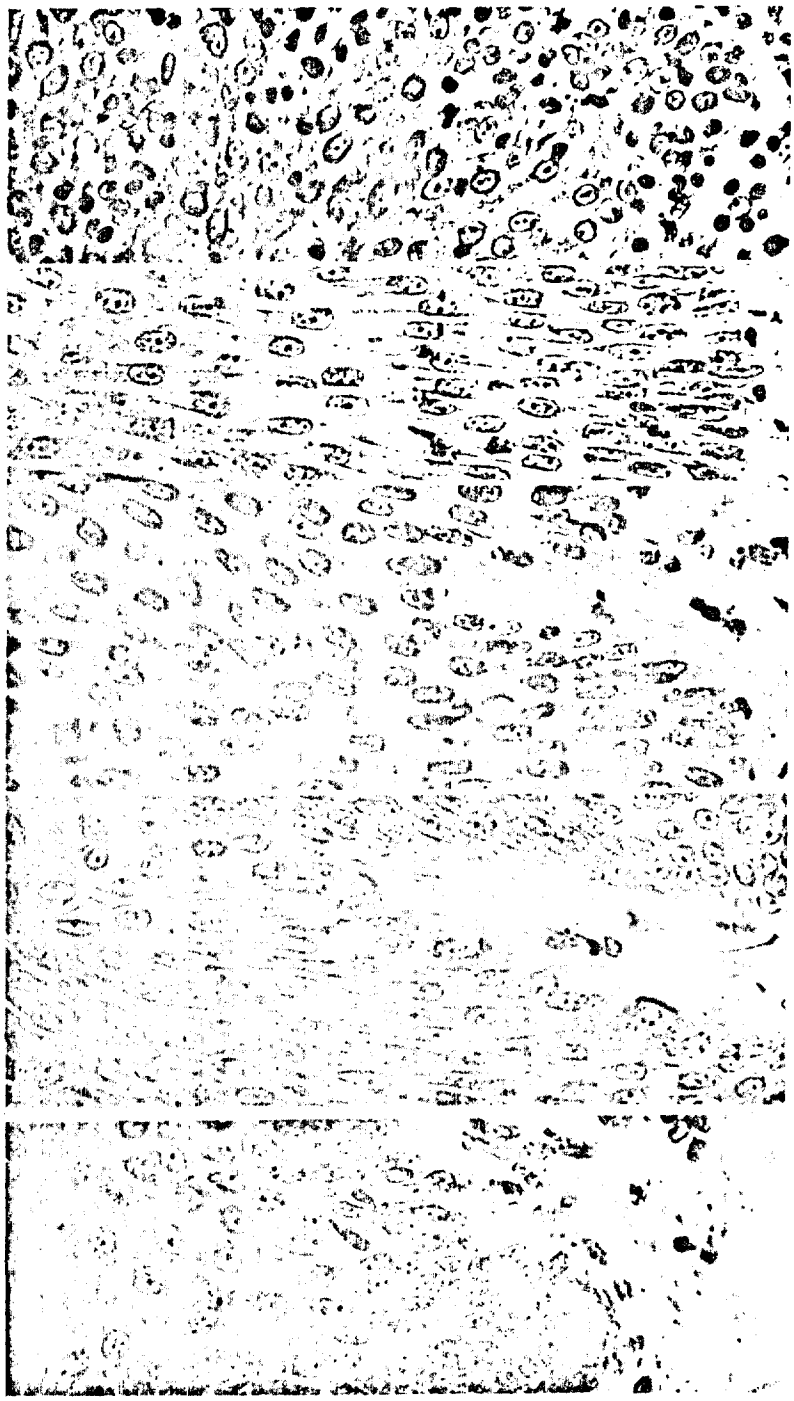

Fig. 3.—Higher power of Fig. 2 shows the transformation of the epithelium from normal in *A* to almost complete breakdown in *E*. Note the crowding of the nuclei in the germinal layer in *B* and the contrast of spacing and arrangement in vertical rows in *D*. (×514.)

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Fig. 4.—Same as Fig. 1. Higher power of corium. The corium in *B* is hyperplastic with more numerous fibroblasts. In the succeeding stages, the edema and the presence of inflammatory cells became progressively more marked. (×220.)

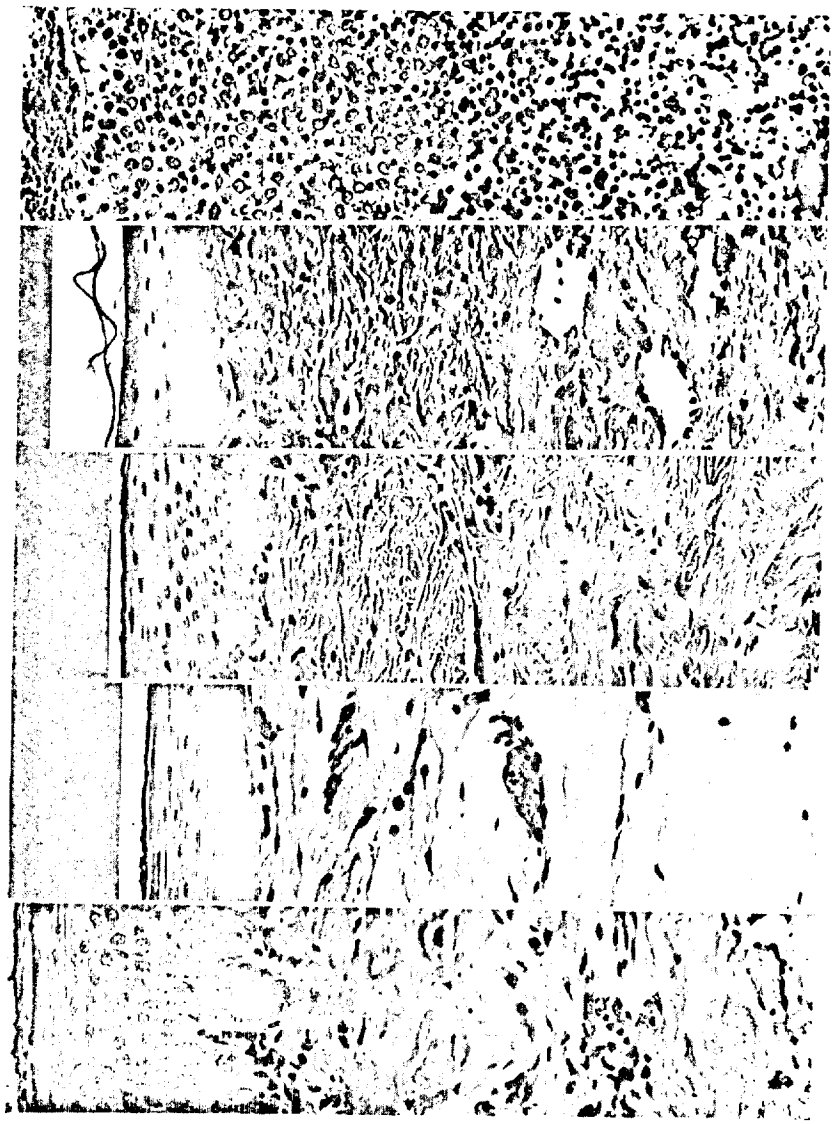

Fig. 5.—Same as Fig. 1. Areolar gingivae. The normal mucous membrane contains no keratin layer (*A*). In *B*, a keratin layer is seen which persists through *C* and *D*. In *B*, the epithelium appears compressed as the connective tissue becomes more compact. In the remaining stages, the epithelium becomes thinner as the edema in the corium increases. Note the increased number of fibroblasts in *B* in comparison with the preceding stage and the succeeding stages. (×286.)

a more vertical arrangement. (Figs. 2, *C* and 3, *C*.) Certain beginning degenerative changes were noted. The upper half of the prickle cells showed mild intercellular and intracellular edema with the bridges slightly obscured. The keratin layer no longer presented a firm even contour but appeared to be either totally lost or in a process of desquamation. The corium showed edematous areas infiltrated by a considerable number of inflammatory cells (Fig. 4, *C*).

The biopsy made after nine months of treatment disclosed primarily degenerative changes in both the epithelium and connective tissue. The prickle cells were arranged in a vertical pattern, and the crowding of nuclei and splitting of pegs were not seen. The keratin layer was almost absent and the intercellular edema had increased. (Figs. 2, *D* and 3, *D*.) The corium showed marked increase in inflammatory involvement. The cellular invasion consisted chiefly of round cells with occasional plasma cells and polymorphonuclears. The edema was widespread (Fig. 4, *D*).

The thirteenth month or post-mortem gingival section showed almost total degeneration. Little keratin was noted. The epithelial elements were indistinct with widespread edema and inflammatory cell invasion. Necrosis and consequent loss of portions of the epithelial layer were in evidence. The entire section appeared fragmented. The corium seemed to consist mainly of inflammatory cells and edema. Collagenous elements were either minutely fragmented or completely displaced and obliterated by the exudate. The inflammatory cells were primarily of the round-cell variety with occasional plasma cells and immature polymorphonuclears. The necrotic tissue was particularly characterized by the paucity of polymorphonuclears. (Figs. 2, *E*, 3, *E*, and 4, *E*.)

Mucous Membranes.—The mucous membranes followed a similar pattern of change in some respects as the gingivae, with differences (Fig. 5). The normal mucous membranes do not possess a keratin layer (Fig. 5, *A*). However, in the first experimental biopsy, a definite layer of keratin was seen (benign metaplasia) (Fig. 5, *B*). The epithelium was compressed, probably owing to the increase of fibroblasts and collagenous elements in the corium. The corium appeared more compact. In the next stage (after six months of treatment), the beginning breakdown process became apparent (Fig. 5, *C*). The keratin layer was still present, but edema was evident in both the epithelium and connective tissue. At the end of the nine-month period, the keratin was seen stripping off, the edema had increased, and atrophy of the epithelium was seen (Fig. 5, *D*). In the final stage, the breakdown appeared almost complete, with total absence of keratin, almost complete atrophy of the epithelium, and marked cellular infiltration of the entire tissue with areas of necrosis (Fig. 5, *E*).

Vagina.—Detailed histologic study has not yet been completed. The changes noted thus far bore similarity to those seen in the gingivae. Edema and necrosis were the most common. We report here the findings in one dog showing loss of surface epithelial cells, atrophy, and complete destruction of portions of the epithelium. (Figs. 6 and 7.)

Uterus. The uteri of the dogs varied histologically, but in degree rather than in kind. In those cases where the uterus was mildly affected, the endo-

Fig. 6.—Dog No. 736. Vagina. Estrogen implants only. Maximum daily absorption 220 μg daily. Duration, forty weeks. (×23.) *A*, Loss of surface cells from epithelial lining of vagina. *B*, Atrophy and complete destruction of portions of epithelium.

Fig. 7.—Higher power of Fig. 6, showing hydropic degeneration of epithelial cells with desquamation at *A* and atrophy with complete loss of epithelial cells at *B*. (×170.)

metrium appeared to be the only site of activity. The lumen usually contained necrotic tissue debris and desquamated epithelial cells. The normal arrangement of the uterine glands was distorted and there was destruction of the epithelial lining cells. The involvement extended almost to the myometrium. Edema and inflammatory cell infiltration were consistently present. The cellular exudate varied from a predominantly polymorphonuclear reaction with localized small abscess formations to a purely round-cell involvement with very few normal mature polymorphonuclears. Small isolated hemorrhagic areas were visible in some instances.

The moderately involved uteri presented a picture basically comparable to the foregoing, but the affected areas were more extensive. The necrosis of the endometrium was far-reaching with few normal uterine glands visible. Hemorrhage, debris, edema, and inflammatory cell invasion were more extensive. In addition, the myometrium was involved. The muscle layers showed inflammatory cell infiltration and edema. Necrosis was present here also in some cases. The character of the cellular exudate in the endometrium and myometrium appeared uniform.

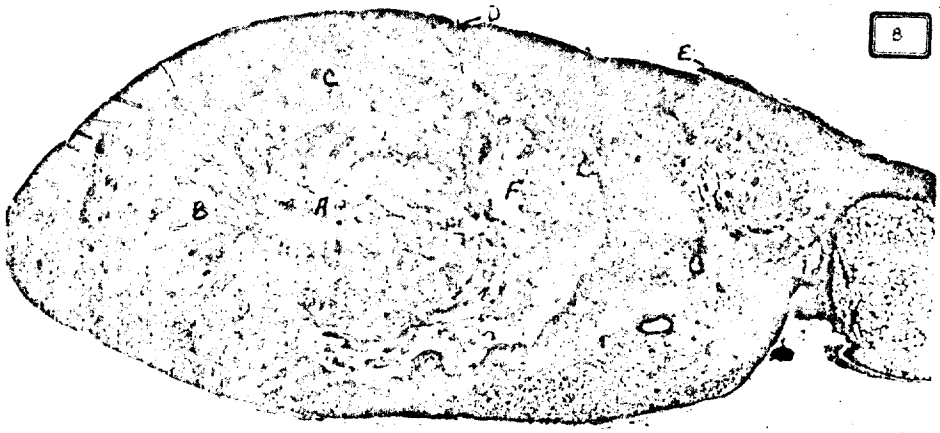

Fig. 8.—Dog No. 736. Uterus. Estrogen implants only. Maximum daily absorption, 220 micrograms. Duration, forty weeks. (×16.) *A*, Lumen filled with necrotic debris. *B*, Widespread necrosis and breakdown of endometrium. *C*, Inflammatory involvement and necrosis of myometrium. *D*, Perforation of uterus. *E*, Peritonitis. *F*, Liquefaction necrosis.

In the severely involved uteri, the endometrium was usually totally destroyed. The lumen was barely recognizable, with widespread necrosis and liquefaction of debris throughout. Bacterial masses were observed in all layers of the uterus. The myometrium was similarly affected. In a few cases, large abscess areas were seen with numbers of acute inflammatory cells; in others, the massive necrotic areas were completely devoid of polymorphonuclears. Hemorrhage was a frequent finding. (Figs. 8, 9, 10, and 11.)

In a majority of the dogs in group I, perforations through the serosa and peritoneum were observed. The quality of the peritonitis was consistent with the other microscopic findings, that is, necrosis of tissue and paucity of polymorphonuclears.

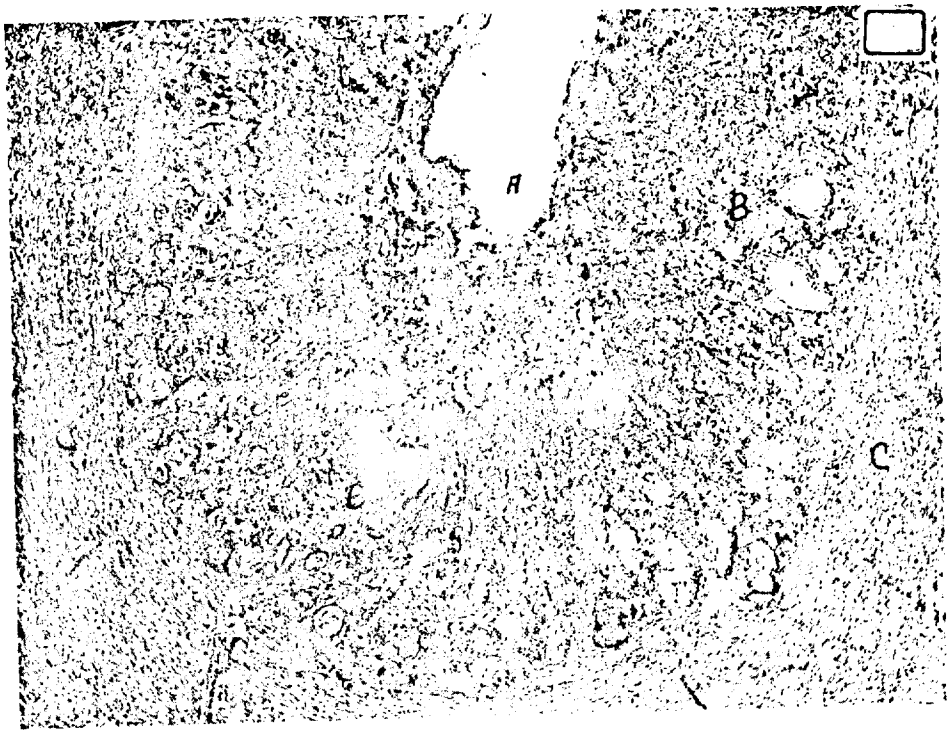

Fig. 9.—Same as Fig. 8. Higher power of endometrium showing *A*, lumen with necrotic debris; *B*, endometrial breakdown with inflammatory infiltration; *C*, myometrium with inflammatory cell infiltration. (×49.)

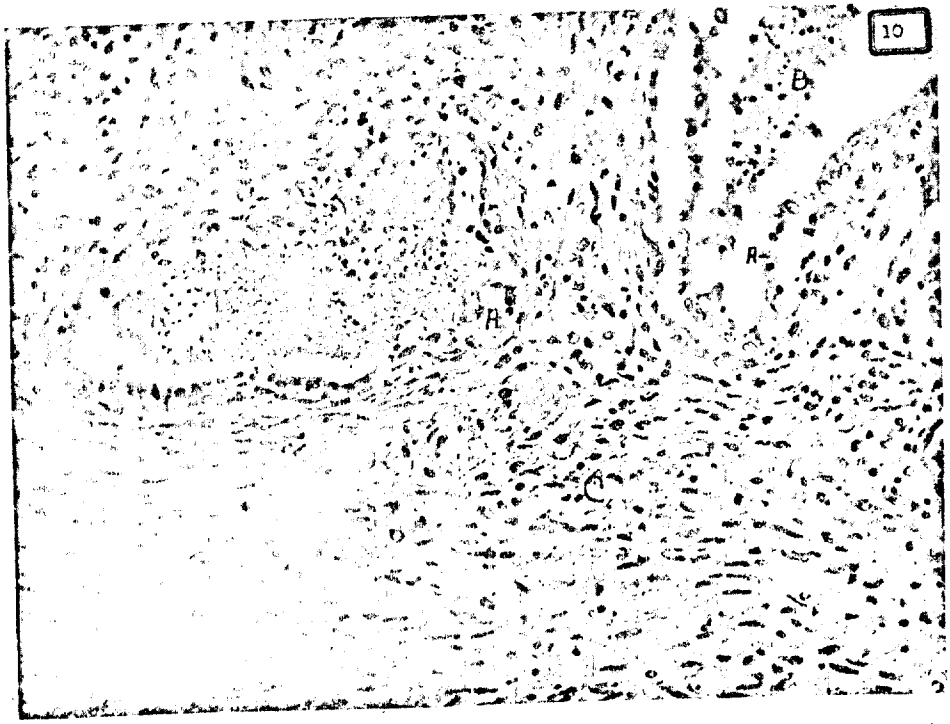

Fig. 10. Same as Fig. 8. Higher power of endometrium and myometrium showing (*A*) destruction of epithelial cells of the uterine glands with (*B*) necrotic debris in the lumina; (*C*) myometrium with inflammatory cell invasion. (×390.)

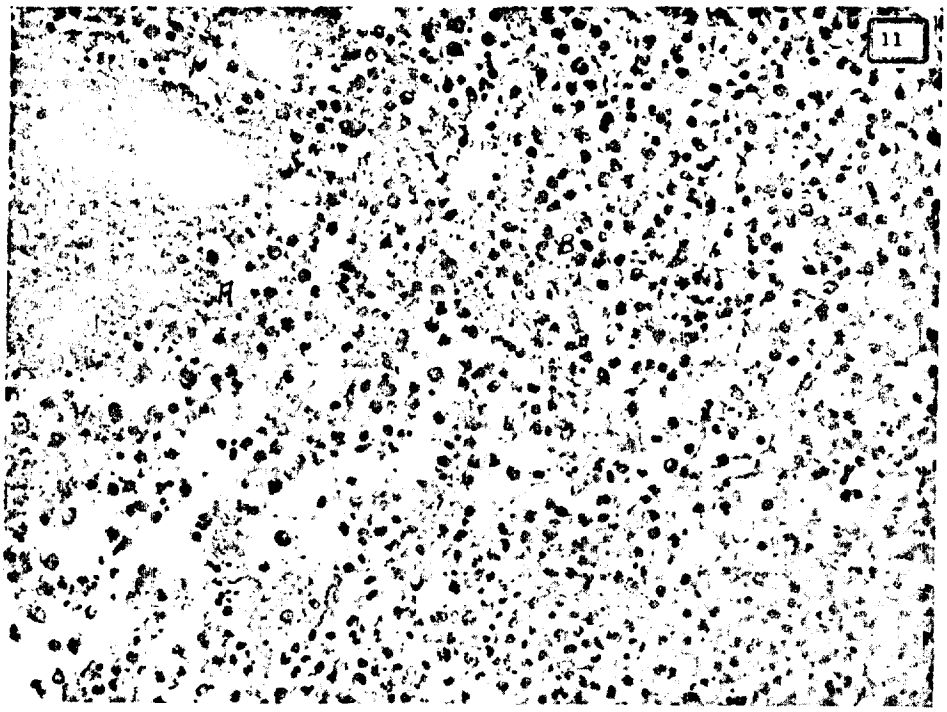

Fig. 11.—Same as Fig. 8. Higher power showing (A) necrosis with liquefaction and a considerable number of immature polymorphonuclears. (×514.)

Fig. 12. Dog No. 736. Humerus. Same animal as in Fig. 8. The bone marrow has been almost totally replaced with fatty tissue. No hemopoietic activity is observed. (×18.)

Bone Marrow.—Bone marrow studies are incomplete. Where studied, complete fatty replacement was seen (section of humerus) (Fig. 12).

Discussion.—

At autopsy, it was found that the most severe necrosis had developed in the uterus. The gingivae were the next most seriously involved and then the vagina. The other organs were not uniformly affected. Terminally, the dogs refused food and died or were sacrificed as a result of acute peritonitis or anemia.

The most marked anemias were observed terminally, although in some instances the symptoms came on within a month after implantation. The most severe blood affection seen was a marked leucopenia. The red blood cell change was not as great. The hemoglobin was least involved. The bone marrow was seriously altered; in some instances necrosis was noted, in others, complete fatty replacement.

Those animals receiving the soybean lecithin were in some degree protected against the toxic symptoms. Although skin lesions developed, they came on later in the experimental period and were not as severe. The lecithin seemed to afford the most protection to the uterus. In three cases, no changes were observed in the uterus while in the group not being fed lecithin all indicated moderate or severe alterations. The three dogs receiving lecithin from the beginning showed the least involvement. This finding is in agreement with that of Foldes and Murphy, who reported a neutralizing action of toxic effects between lipolecithin and certain steroids.

In comparing the effects of the estrogen implants in dogs with our former experiments in estrogen-injected monkeys, we found no such toxic effects in the monkeys even when large doses were applied. In fact, the results of the present study, as well as others employing dogs as experimental material, demonstrate a destructive effect of the drug, while similar investigations on monkeys and rats, with even larger doses of the same hormone, have produced reactions of a proliferative nature. The significant questions arising out of this conflict are: (1) whether or not the disagreement may be attributed to the difference in species and (2) whether endogenous estrogen in humans, when present in large amounts, may in some measure be responsible for the degenerative affections, similar to those described in dogs, observed in human gingival and other tissues.

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